UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

"Evaluation of faecal sampling methods for the analysis of *Giardia* sp. in companion animals"

Ana Rita Ruas dos Santos

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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“All our dreams can come true, if we have the courage to pursue them.”
Walt Disney

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To my Father, Mother and Syster: thank you for everything. I hope you're proud dad!

To Jon Jon MC, for all the love...."alone we go faster, but together we go further!"

"Evaluation of faecal sampling methods for the analysis of *Giardia* sp. in companion animals"

Abstract

Giardiosis is a zoonosis with a worldwide distribution, responsible for gastrointestinal disease in both humans and animals. Transmitted by contaminated water and food, *Giardia* represents a risk to veterinary and public health. Because of its prevalence in companion animals, veterinarians and animal owners awareness of the importance of its identification and treatment in infected animals, made it an important part of the routine diagnostic techniques in the parasitology laboratories.

In this project, faecal sampling for Immunofluorescence Microscopy technique (IF) was evaluated in order to improve *Giardia* diagnostic routine at the Statens Veterinärmedicinska Anstalt Parasitology Laboratory, Uppsala, Sweden. The objective was to see if there were differences between using the recommended three samples from three different days from the same animal, and using only one sample. It was also evaluated if a protocol procedure change, while subsampling the faeces at laboratory level, could influence the final result.

From 282 animals (dogs and cats) faecal analysis, once again it was proved that the use of three samples is important to improve IF sensitivity, especially in faeces with low cyst concentration, since it returned less false negative samples. The three samples can be analyzed as a pool for there was no difference when analyzed separately. Regarding the technique, the swab should be used in different places of the sample to improve the cysts detection, although it does not increase the sensibility. In accordance with other authors, the IF sensitivity is afected by a low number of cysts in the samples but the clinical relevance of the presence of *Giardia* in these animals should be evaluated through a close communication between the clinical veterinary and the Parasitology Laboratory.

Key-words: *Giardia*, faecal sampling, Immunofluorescence Microscopy, sensitivity, SVA, Sweden.

"Avaliação de diferentes métodos de amostragem fecal para o diagnóstico de *Giardia* em animais de companhia."

Resumo

A giardiose é uma zoonose com distribuição mundial, responsável por causar doença gastrointestinal em animais e humanos. Transmitidos por água e alimentos contaminados, os protozoários do género *Giardia* representam um risco para a saúde pública e veterinária. Devido à sua prevalência em animais de companhia, os veterinários e proprietários dos animais consciencializaram-se da importância da identificação e tratamento de animais infectados, integrando-a nos diagnósticos de rotina dos laboratórios de parasitologia.

Neste projecto, foi avaliado o método de amostragem fecal no sentido de melhorar a técnica de Microscopia de Imunofluorescência (IF) usada como diagnóstico de rotina no Laboratório de Parasitologia do Instituto Statens Veterinärmedicinska Anstalt, Uppsala, Suécia. O objectivo foi avaliar as possíveis diferenças entre usar três amostras de três dias diferentes para um mesmo animal, e usar somente uma amostra. Foi também avaliado se uma alteração num dos procedimentos do protocolo, relativamente à subamostragem a nível laboratorial, poderia influenciar o resultado final.

De 282 animais (cães e gatos) testados a nível coprológico, uma vez mais se concluiu que o uso de três amostras é importante para melhorar a sensibilidade da IF, nomeadamente em amostras com baixa concentração de quistos, uma vez que apresenta menos falsos negativos. As três amostras podem ser analisadas em "pool" pois não se verificaram diferenças relativamente à análise individual. A colheita de subamostras com a zaragatoa deve ser efectuada em diferentes locais da amostra de fezes para facilitar a observação dos quistos, ainda que não aumente a sensibilidade do método. Tal como demonstrado por outros autores, a sensibilidade da IF foi afectada pelo baixo número de quistos nas fezes mas a relevância clínica da presença de *Giardia* nestes animais deverá ser avaliada através da cooperação entre o Veterinário clínico e o Laboratório de Parasitologia.

Palavras-Chave: *Giardia*, amostragem fecal, Microscopia de Imunofluorescência, sensibilidade, SVA, Suécia.

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List of Abbreviations and Symbols

Cl^- – Chlorum
DFAT – Direct Fluorescent Antibody Test
DV – District Veterinarians
EIA – Enzymatic Immunoassay
ELISA - Enzyme-linked Immunosorbent Assay
ESCCAP – European Scientific Counsel Companion Animal Parasites
FE – Formalin-ether
FECM – Formol-ether Concentration Method
FEA – Formolethylaceate
FMV – Faculdade de Medicina Veterinária
FN – False negatives
GI – Gastrointestinal
ICZN - International Code of Zoological Nomenclature
IBD – Inflammatory Bowel Disease
IBS – Irritable Bowel Syndrome
IF – Immunofluorescence Microscopy
IMS – Immunomagnetic Separation
Leg. – Legitimate
Na^+ – Sodium
PBS – Phosphate buffered saline
PCR – Polymerase Chain Reaction
sp. – specie
spp. – species
SPG – Specific Gravity
SPSM - Sub-pixel Sweeping Microscopy
SSF – Sheather's Sugar Flotation
SVA – Statens Veterinärmedicinska Anstalt (National Veterinary Institute)
SVAPL - SVA Parasitological Laboratory
TN – True negatives
TP – True positives
ZSCT – Zinc Sulphate Concentration Technique
$ZnSO_4$ – Zinc Sulphate

I. TRAINEESHIP DESCRIPTION

The curricular traineeship was accomplished in Sweden under Erasmus Program, in two different institutions, for a total period of 8 months. The first 6 months (from September 2015 till March 2016) were accomplished at District Veterinarians in Värmland, with a total amount of 1320 hours, under the supervision of Börje Adolfson (Clinical Manager) and Inger Olsson (Leg. Veterinary). From April to May, the training was performed at the National Veterinary Institute (SVA), in Uppsala, with a total amount of 360 hours, supervised by Dr. Eva Osterman (Head of Section for Parasitological Diagnostics at SVA) and co-supervised by Professor Doctor Luís Manuel Madeira de Carvalho (FMV- Lisbon University).

1. Activities at District Veterinarians (DV)

At DV, was possible to acquire scientific and practical skills regarding both small animal practice (dog and cat) and farm animal practice (ruminants, horses and pigs), either in routine operations, or in the resolution of acute situations. It included different fields such as animal health, prevention of infectious and parasitic diseases, herd health, productive and reproductive management of farm animals.

2. Activities in the National Veterinary Institute (SVA)

At the Parasitological Laboratory in SVA, was possible to learn different diagnosis techniques in order to identify different parasites. The main method used was immunofluorescence microscopy (IF), in cat and dog faeces, to evaluate the presence of *Giardia* and *Cryptosporidium*.

With the same goal, two other techniques were also tested for educational purposes. Samples found to have high amount of cysts (in the IF) were used to test the Mini-FLOTAC® device. If proven to be accurate, it could be an easy and quick method to evaluate the infection with *Giardia* cysts by only using 2 grams of faeces. The Zinc Sulphate Concentration Technique (ZSCT) was used in samples with a very low amount of cysts, after being analysed with IF, in order to compare the efficiency of both methods in diagnosing giardiosis in mild infections. Both methods were only performed in a small number of samples, so the results cannot be used to draw valid conclusions.

To identify other parasites in faeces, both flotation with sugar/salt solution, to evaluate intestinal nematodes, helminths and coccidian oocysts, and Baermann Funnel Technique, to evaluate the presence of lungworms (*Crenosoma vulpis, Oslerus osleri, Eucoleus aerophilus)* and french heartworm (*Angiostrongylus vasorum*), were performed.

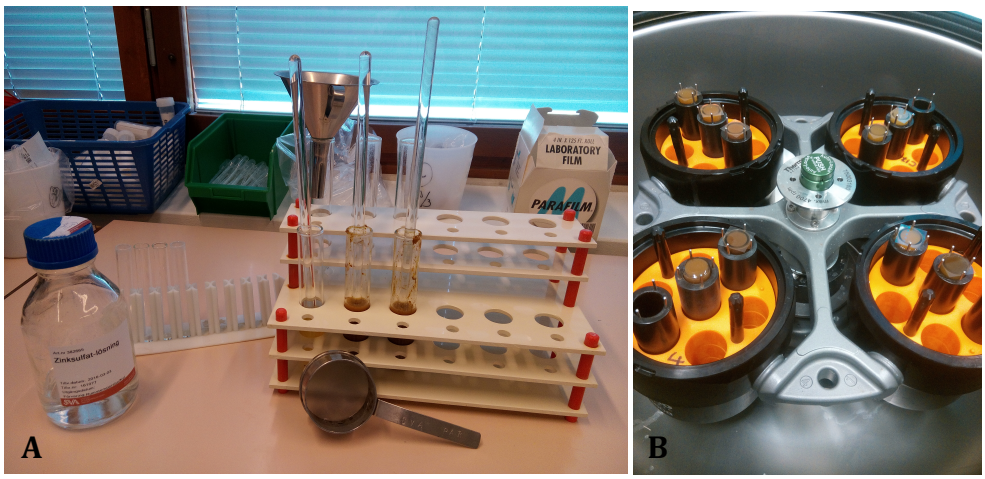
As the traineeship was accomplished in April and May, many samples of horses were being sent for analysis. In order to help in the laboratory, faecal cultures were performed for gastrointestinal parasites nematode larvae to L3 and also the Modified McMaster method, to count strongylid eggs (eggs per gram, EPG). A brief description of some of the techniques is presented next to give an overview of what was done.

Faecal Flotations (ZSCT and Sugar/Salt)

Faecal flotation has been used to diagnose most of dogs and cats gastrointestinal parasites due to its ability to recover eggs, cysts and oocysts, based on differences in the specific gravity of eggs, fecal debris and flotation solution (Katagiri & Oliveira-Sequeira, 2010).

For this method, 2 to 5 grams of faeces were homogenized with 15ml 33% zinc sulphate solution (or sugar/salt solution, depending on the parasites), strained through a sieve and the liquid was collected into a 10ml centrifuge tube (figure 1A). The tube was filled with 33% zinc sulphate solution until a positive meniscus was clearly observed at the top of the tube. A cover slip (22x22) was placed on the top of the meniscus (figure 1B), and centrifuged 5 minutes at 1000 rpm. The cover slip was carefully removed and placed on a slide to be examined under an optical microscope.

Figure 1 – ZSCT: preparation of the samples (A) and centrifugation step with cover slide on the top of the tubes (B). Original.



After the observation of eight positive animals (each with 3 different samples), it was understood that, despite this method concentrates the cysts, increasing the chances of a positive diagnosis, there are some disadvantages to be considered. As *Giardia* cysts are hard to find, because they look like a shadow in the middle of the debris and other parasites, it requires an experienced and trained technician. It is also hard to define at what level of the

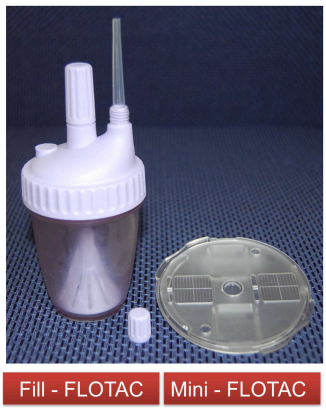
sample they are and sometimes they can be confused with other existing forms, which can lead to a false positive. False negatives can also occur if the technician fails to find them, and also if the cysts are distorted due to the zinc sulphate action. This method procedure is easy and fast to perform, but the observation takes a long time if the number of cysts is very low. As the zinc sulphate destroys the cysts, the slide observation should be done as soon as possible after preparation. After some time (not yet defined), is not possible to ensure if the cysts are still unchanged.

Mini-FLOTAC®

Mini-FLOTAC® (figure 2) device was created after FLOTAC® to allow all laboratories to use it without being required special equipment as it is for FLOTAC®, such as a centrifuge with an adapter for the device. It is a method with an easy and fast procedure, but not described as a diagnosis method for *Giardia* (B. Barda, Ianniello, et al., 2014).

To test this method for *Giardia* identification, faecal samples with high amount of cysts, previously observed in the IF technique, were chosen to guarantee the presence of *Giardia* cysts in the reading disc. Two grams of faeces were homogenized with 38ml of zync sulphate solution and filtered using the Fill FLOTAC® (figure 2).

Figure 2 - Fill-Flotac and Mini-Flotac device (B. D. Barda et al., 2013).



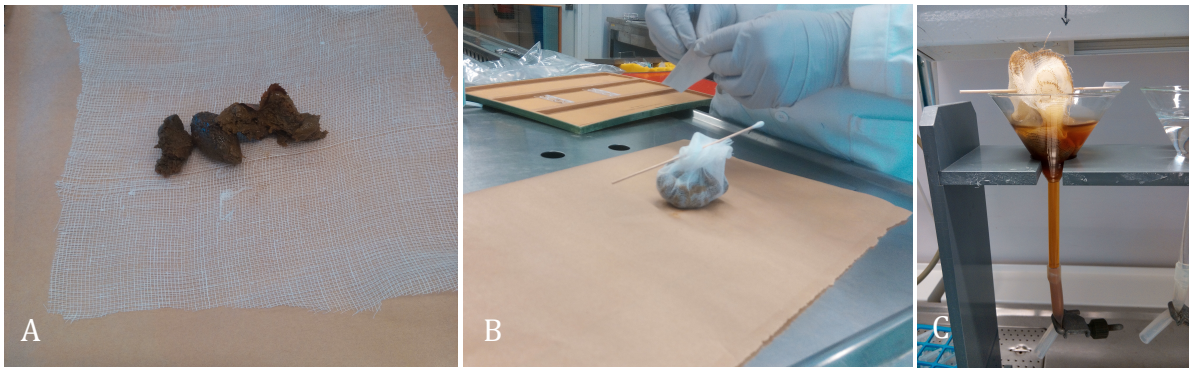
The flotation chambers were filled with 1ml each. The reading discs were observed after 10 minutes in the microscope. From the two analysed samples, only one had a clean preparation that could allow the observation of *Giardia* cysts. The second sample had a high amount of debris, which impaired the observation. As an aggravating, the reading disc of this device is made of plastic and therefore it does not give a clear observation needed for cysts identification. Another negative aspects are the small amount of faeces and the high dilution factor that decreases the chances of finding the cysts. Although the procedure is of easy

execution, the observation of the entire sample is very time-consuming and the cysts identification proved to be very difficult. Nevertheless, this device should be the subject of further studies concerning *Giardia* diagnosis.

Baermann Funnel Technique

The Baermann Funnel technique is the most used method to detect the first stage larvae of lungworms (Bauer et al., 2010). It gives a qualitative analysis based on the larvae migration downward in the water of the funnel, settling in the tube just above the clamp. To perform it, faeces from three days are mixed and about 5 grams are placed in gauze (figure 3A) at the interior rim of the funnel and sealed with a swab (figure 3B). The funnel is filled with tap water at room temperature until half of the sample is submerged (figure 3C). After 12-24 hours, the clamp is taken away and a small amount of water containing the living larvae is collected to a tube. After centrifugation and transfer of the sediment to a slide, the larvae are observed in the microscope.

Figure 3 - Baermann Test. (A and B) Sample preparation for further colocation in a suspended funnel with clamped tubing attached (C). Original.



Culture of GI parasites nematode eggs till L3 larval stages

This method aims to identify different larvae from horse's faeces, like bloodworm, e.g. *Strongylus* spp. and small strongyles, e.g. Cyathostominae, giving a qualitative (genus/species diagnosis) and semi-quantitative analysis (counting 100 different L3 to assess the percentage of genera and species). To promote nematode eggs to develop till L3 larval stages, the faeces are incubated in a glass jar at 27ºC for 10 days. Afterwards, the glass jar is filled with water and a Petri dish is placed on top and both are inverted, so that the larvae are allowed to migrate into the water filling the Petri dish for 24 hours (figure 4). The water is collected with a pipette, centrifuged and the supernatant is discarded. The remaining is used to prepare the slide for observation, fresh or stained with lugol iodine.

Figure 4 – Glass jar in a Petri dish, filled with water. Original.

II. INTRODUCTION

Giardiosis is a gastrointestinal disease affecting companion animals (Baneth et al., 2015). In Sweden, not only animals with clinical signs (up to 13,04% of the dogs and 9,68% of the cats) but also healthy dogs (2,6%) are infected with *Giardia* (Victorsson, 2015; SVA, 2016) so it became an important part of the SVA Parasitology Laboratory (SVAPL) diagnosis daily routine. However, it is *Giardia* zoonotic character that makes it of great importance for public and veterinary health, and infections in humans were already confirmed in Sweden, whom shared the same assemblages identified in animals (Svenungsson et.al, 2000; Lebbad et al., 2010, 2011). This enhances the importance of a reliable but also economic and fast diagnostic method, reason why *Giardia* protozoa and the IF technique used in SVAPL for its diagnosis, were chosen for this research study, aiming the evaluation of the method sensitivity and the improvement of the technique itself, concerning both time and costs associated.

III. LITERATURE REVIEW

1. The Parasite Overview

Giardia is an ubiquitous enteric flagellate protozoan parasite causing intestinal infection in humans, wildlife and domestic animals, such as livestock, dogs and cats (Conboy, 1997; Baneth et al., 2015). This water-borne gastrointestinal parasite is considered the main cause of non-viral diarrhoea. Giardiosis is a zoonotic disease and, therefore, a serious veterinary and public health problem. The zoonotic risk is enhanced by a high prevalence of *Giardia* in dogs (Khan, Mergani, Mohammed, Bano, & Khan, 2014; Kulakova et al., 2014; Pitães, Nunes, Fernandes, & Madeira de Carvalho, 2015). It is also considered the most common cause of outbreaks associated with water intake, with 20% to 30% prevalence in developing countries, where the children infected can have their development and growth compromised, poor cognitive function and even death (Gomes et al., 2011).

Giardia is responsible for 280 million symptomatic infections in humans each year, impairing development and socioeconomic improvements, reason why, in September 2004, was included in WHO "*Neglected Disease Initiative"* (Pitães et al., 2015). All diseases included in this initiative have a common link to poverty (Savioli, Smith, & Thompson, 2006).

Giardiosis is also known as "Beaver Fever", "Backpacker's Diarrhoea" or "Traveler Diarrhoea", because beavers, rodents from North America, are the main reservoir host for this protozoan, favouring *Giardia* transmission by building natural dams and shedding its cysts in

the water, making people who drink water from streams or rivers at higher risk of being infected (Rajurkar, 2012).

2. Taxonomy

Giardia taxonomy has been quite controversial with different names being often used to the same species. If we consider the old systematic based on morphology, this protozoan belongs to subkingdom Protozoa, phylum Sarcomastigophora, subphylum Mastigophora, class Zoomastigophorea, order Diplomonadida and family Hexamitidae (Gillespie & Pearson, 2001). However, the new systematic based on genetic, structural and biochemical data places *Giardia* in phylum Metamonada, subphylum Trichozoa, superclass Eopharyngia, class Trepomonadea, subclass Diplozoa, order Giardiida and family Giardiidae (Plutzer, Ongerth, & Karanis, 2010).

The *Giardia* sp. responsible for human infection was first discovered by Antony van Leuwenhoek in 1681, when he analysed his own stool and described both trophozoites and cysts. In 1859, Lambl described it in more detail naming it as *Cermomonas intestinalis*, and the genus was named *Giardia* in 1883 after Prof. A.M. Giard of Paris. In 1915, Charles Wardell Stiles renamed it *Giardia lamblia* (Payne & Artzer, 2009; Rajurkar, 2012). In the 1970s, the first major reviews about *Giardia* were published, after a large water-borne outbreak of giardiosis during the 1965 winter in Colorado (Gillespie & Pearson, 2001).

Currently, seven *Giardia* species are accepted: *Giardia agilis* (in amphibians), *Giardia ardeae* (great blue heron), *Giardia muris* (in mice), *Giardia microti* (in rodents), *Giardia psittaci* (in birds), *Giardia peramelis* (in quenda) and *Giardia duodenalis* (syn. *Giardia lamblia* and *Giardia intestinalis*). *G. duodenalis* has the broadest host range and greatest public health significance (Bowman, 2014; Baneth et al., 2015; Hillman et al., 2016).

Using polymerase chain reaction (PCR) amplification followed by DNA sequencing, it was possible to identify eight genetic assemblages (A-G) from *G. duodenalis* (Bond et al., 2015). These assemblages might correspond to different species and are distinguishable only based on genetic polymorphisms (and not on morphology). Different names were proposed for each, even though they need to be re-described and their names validated with biological and molecular data in compliance with the International Code of Zoological Nomenclature (ICZN) so that they can be accepted as valid species (Ryan & Cacciò, 2013). Assemblages A (*G. duodenalis*) and B (*G. enterica*) are zoonotic, supported by their occurrence in human and animal hosts (for example dogs) in the same geographical areas (Ehsan et al., 2015). Assemblages C and D (also defined as *G. canis*) are exclusively from dogs, assemblage E is from artiodactyls (*G. bovis*), assemblage F from cats (*G. gati*), assemblage G from rats

(*G. simondi*) and assemblage H from seals (Fiechter, Deplazes, & Schnyder, 2012; Zheng et al., 2015).

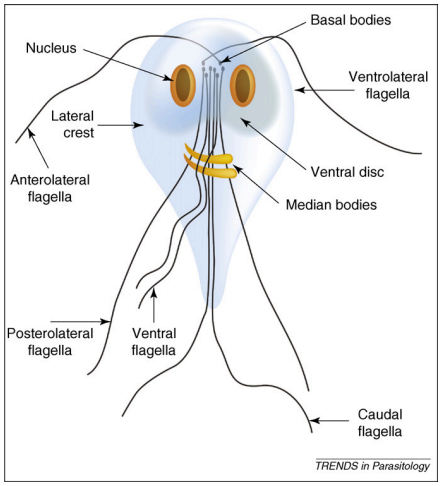
Assemblage B is the most variable, which causes problems with data interpretation during molecular diagnostics (Stojecki, Sroka, Cencek, & Dutkiewicz, 2015).

Assemblage A can also be divided in four sub-assemblages, AI, AII, AIII and AIV, based on protein polymorphisms of 23 loci. AI e AII are related with human hosts, while AI, AIII and AIV are found in animals (Ryan & Cacciò, 2013).

3. Morphology

Giardia can be found in two different forms: the trophozoite and the cyst. Trophozoites are adapted for attachment to the mucous epithelial cells of the small intestine. They are shaped like a teardrop, with a sucking disc on the anterior ventral side and two symmetric nuclei with a large endosome each (figure 5) (Bowman, 2014).

Figure 5 - *Giardia duodenalis* trophozoite (Monis, Caccio & Thompson, 2009).



As other subcellular structures are described two slender axonemes, four pairs of flagella (anterolateral and ventrolateral, posterolateral, ventral and caudal) and a pair of median bodies (Bowman, 2014). It lacks some organelles typical from higher eukaryotes, such as mitochondria, peroxisomes and a typical Golgi apparatus (Gillespie & Pearson, 2001).

In the small intestine, the trophozoites attach to the mucosal cells by their sucking discs, microtubule, contractile protein activity and by a mannose binding lectin, unlike other intestinal flagellates that are found in the cecum and colon. Trophozoites usually form infective cysts before passing out with the feces, as an adaptation for survival outside the host (Rajurkar, 2012; Bond et al., 2015).

The cyst (figure 6) is oval, around 11-14µm in length and 7-10µm in width and contains 4 nuclei with a rigid outer wall that protects it from extreme conditions, like temperature and chlorination (Bond et al., 2015). In permanent staining, it might be seen a "halo effect" outside the cyst wall due to the shrinkage caused by dehydrating agents (Rajurkar, 2012).

Figure 6 - *Giardia* cysts stained with Lugol's iodine with nuclei (N), intracytoplasmic flagella (F), and median bodies (M) (Zajac & Conboy, 2012).

A mature cyst, containing two potential trophozoites, is the form usually found in the faeces of infected hosts. The ingestion of only ten cysts is enough to lead to infection. Although trophozoites may also be passed, especially with diarrheal stools, they are incapable of causing infection and soon die, especially if they go into fresh water, suffering lyse due to their inability to osmoregulate (Bowman, 2014).

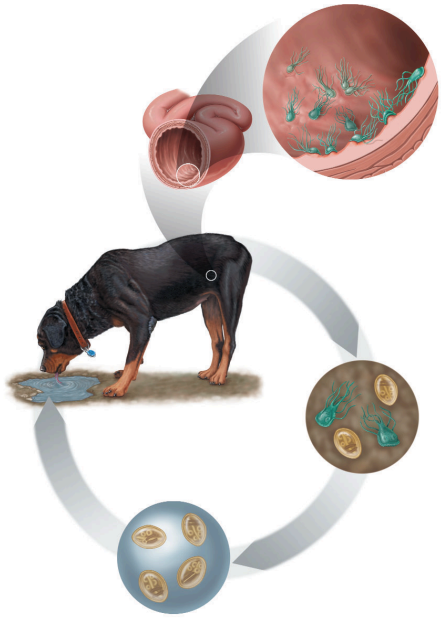
4. Life Cycle

The life cycle of *Giardia* (figure 7) is direct and combines two stages: an environmentally resistant infectious cyst form, the infective stage, and a motile trophozoite, the replicative stage (Bond et al., 2015). *Giardia* virulence is dependent on both parasite and host factors, and can cause disease with only ten cysts. The cyst is immediately infectious when released into the faeces and can remain infectious for months in cool moist areas, accumulating in the environment. When ingested by the host, the cyst becomes metabolically active and excystation occurs within as little as 15 minutes by action of gastric acid, cysteine proteases produced by *G. duodenalis* peripheral vesicles, phosphorylation/dephosphorylation of cyst wall proteins and Ca^{2+} signaling. The released excyzoite undergoes two rounds of binary fission while upregulating processes related to mobility and the organization of the adhesive disc. Epidemiological and molecular genetic studies has shown that, besides asexual reprodution by simple binary fission, *Giardia* is also capable of sexual reproduction (Monis et al., 2009). In the duodenum, one cyst releases two trophozoites that adhere to the intestinal

enterocytes, causing their apoptosis and diarrhea due to the malabsorption of Na^+ and glucose and to the hypersecretion of Cl^- (Esch & Petersen, 2013). After repeated mitotic division in the gut lumen, environmentally resistant cysts are formed by encystation, as a consequence from trophozoite exposure to alkaline conditions or bile salt. Cysts pass through the intestine in faeces and are spread by contaminated water, food and fomites and also by direct physical contact (Baneth et al., 2015; Bond et al., 2015).

The prepatent period in dogs and cats is from 5 to 16 days, the cyst shedding is often intermittent (Pitães et al., 2015) and the incubation period is about 12-20 days (Rajurkar, 2012). *Giardia* has 3 patterns of excretion: high (when is present in almost all the stool samples of the patient), low (when is present in 40% of all the stool samples of the patient) and mixed (when periods with high excretion alternates with periods with low excretion) (Rajurkar, 2012).

Figure 7 - *Giardia* life cycle (Blagburn, Dryden, & Pfizer Animal Health, 1999).



5. Pathology

Both host and parasitic factors are responsible for *Giardia* malabsorption diarrhoea, even though the mechanisms are not completely understood.

This parasite induces enterocyte apoptosis, associated with disruption of cytoskeletal and tight junctional proteins. Specific receptors activated by thrombin, modulate cell apoptosis and increases enterocyte permeability. This loss of epithelial barrier function can lead to inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) which can also be a

potential complication of microbial-induced disruption of epithelial permeability (Savioli et al., 2006). In chronic giardiosis, the duodenal surface area is decreased due to the diffuse $CD8^+$ lymphocyte-dependent shortening of epithelial brush border microvilli, leading to malabsorption and poor digestion. Malabsorption of nutrients and electrolytes creates an osmotic gradient that draws water into the small intestinal lumen resulting in small intestinal distension and rapid peristalsis, leading to diarrhoea. Parasitic surface molecules, like giardins (alpha, beta, delta and gamma), a complex network of contractile proteins, serine and cysteine proteinases, are believed to be involved in the diarrhoea mechanisms by breaking the epithelial barrier and host inflammatory and immunological responses (Savioli et al., 2006; Buret, 2008). The adaptive immune responses and mast cell degranulation increase the intestinal transit rates leading to diarrhoea (Buret, 2008).

The increased chloride secretion caused by *Giardia* and high levels of undigested carbohydrates (converted into short chain fatty acid by colonic microbiota) should also be considered as a cause of diarrhoeal disease (Buret, 2008).

6. Epidemiology

Giardia is transmitted via faecal-oral route and from contaminated drinking water and food. Three potential transmission cycles can be considered: anthroponotic, zoonotic (animal to human and human to animal) and between dogs (Bond et al., 2015).

It's responsible for a severe intestinal parasitic disease, highly prevalent in regions that lack adequate water management. In endemic areas with high environmental contamination, the reinfection rate can reach 90% (Kulakova et al., 2014).

The incidence and prevalence of giardiosis in humans and animals has been documented worldwide, with great variations among populations and geographic locations. Though asymptomatic giardiosis is frequent, it has been estimated that about 200 million people in Asia, Africa and Latin America have symptomatic infection with *Giardia*, particularly children and immuno-deficient people. It is characterized by diarrhoea, severe abdominal pain, bloating, flatulence, weight loss and malabsorption, with an incidence of 500.000 new cases each year. This symptoms mimic Irritable Bowel Syndrome (IBS), but the gas expelled has a distinctive smell. This is due to the hydrogen sulphide gas produced by *G. duodenalis*, from anaerobic metabolism of cysteine (Rodríguez et al., 2014; Baneth et al., 2015; Bond et al., 2015). Sometimes giardiosis is not recognised because the acute stage lasts only some days and the differential diagnosis with acute viral enteritis, bacterial food poisoning, intestinal amoebiasis or infection with toxinogenic *Escherichia coli* should be considered

(Rajurkar, 2012). Humans are infected by assemblages A and B, being assemblage B more prevalent (58%) then assemblage A (37%) worldwide (Ryan & Cacciò, 2013).

In dogs, *G. duodenalis* is the most frequent parasite with a prevalence between 12.5% to 34.4%, with higher prevalence in young dogs and dogs kept in kennels, shelters and flocks (Mircean, Györke, & Cozma, 2012; Zanzani, Di Cerbo, et al., 2014; Zanzani, Gazzonis, Scarpa, Berrilli, & Manfredi, 2014). Despite most infections are asymptomatic (Pitães et al., 2015), giardiosis has been associated with the occurrence of diarrhoea and illness in puppies and kittens (Baneth et al., 2015). In symptomatic animals the main clinical sign is persistent diarrhoea resulting from intestinal malabsorption, which may begin as early as 5 days after exposure to infection. Cysts first appear in the faeces after a week or two. As a worldwide parasite, *Giardia* prevalence variation in kennel animals (from 1% to 100%), depends on the age of the dog, living conditions, animal density, nutritional and immune status, and the diagnosis methods used (Mircean et al., 2012). Though less prevalent, from 10.1% to 22.8%, in cats *Giardia* trophozoites are found in the jejunum and ileum, instead of the duodenum, and the faeces are often mucoid, pale, soft and malodorous (Bowman, 2014; Zanzani, Gazzonis, et al., 2014).

Although human and dogs share the same *G. duodenalis* assemblages, it is still unclear if the infection is acquired from the contact between them, or if both have a common infection source, such as contaminated water (Baneth et al., 2015).

The same question is made for the possible zoonotic transmission of *Giardia* between cattle and their handlers. Some studies have demonstrated the presence of both *Giardia* and *Cryptosporidium* in cattle, people and water ponds from the same rural area, but no conclusions about the transmission pattern could be made (Ehsan et al., 2015). Although assemblage E generally predominates in cattle, assemblage A detection has increased, suggesting that is probably more widespread in the bovine population than initially assumed. Even more relevant is the fact that, in a study in India, sub-assemblage A1 was found both in farmers and cattle, supporting the possibility of zoonotic transmission. In Canada, was also suggested the possible transmission of *Giardia* cysts between humans and swines, by contaminated water or food, based on the identification of sub-assemblages AI and AII in these animals (Ryan & Cacciò, 2013).

Several surveys showed that carnivore pets host-specific (C, D, F) and zoonotic assemblages (A and B) of *G. duodenalis* prevalence values, strongly depend on the diagnostic. Other zoonotic parasites such as *Toxocara canis* and *T. cati, Trichuris vulpis* and Ancylostomatidae, are also often found. This highlights the veterinarians role in educating pet owners in order to avoid potential zoonotic risks (Zanzani et al., 2014).

Studies also concluded that, in canine samples collected from public and veterinary health institutions across Europe, 68% were either assemblage C or D (the most prevalent), 23% were assemblage A and 9% were assemblage B. In Sweden, 96% were typed as either assemblage C or D, or both (McDowall et al., 2011).

In contrast, feline faecal samples more frequently contain the potentially zoonotic assemblages A and B of *G. duodenalis*, with assemblage A as the most prevalent and assemblage B found in a much lower percentage. Assemblage F is also found in feline samples (McDowall et al., 2011). This emphasizes the epidemiological role of cats in transmission of giardiosis to humans, and the importance of an accurate diagnosis and treatment of this disease (Paoletti et al., 2011).

Besides dogs and cats, cattle, sheep, pigs, horses, goats and some wildlife species are defined as natural hosts for *G. duodenalis* assemblage A, while cattle, sheep, horses and also some wildlife species are defined as natural hosts for *G. duodenalis* assemblage B. Both assemblages have also been detected in some marine animals such as dolphins, porpoises, seals, common eiders, and thresher shark, which don't represent a direct zoonotic risk, but can contaminate water used by humans for recreation activities. Flies (7,3%) and wild birds (5–49% in faecal droppings) should be considered as vectors for *Giardia* cysts for both assemblages A and B (Plutzer et al., 2010).

Giardia in Sweden

In Sweden, human giardiosis was notified in 1989 and about 1500 cases have been reported annually. In a study, 2% of the human patient with diarrhoea were infected with *Giardia* cysts (Svenungsson et al., 2000).

Giardia zoonotic risk in Sweden was considered after the identification of the same assemblage A in a few human infections, Swedish cats and ruminants, and after identification of assemblage B in exotic pets (such as rabbit and guinea pig) (Lebbad et al., 2010, 2011).

In 2013, from the companion animals with clinical signs tested at SVA, approximately 8% of the dogs and 9% of the cats were positive for this parasite (SVA, 2016). However, adult healthy dogs (over one year old) can also be positive to *Giardia,* with a prevalence of 2,6%, even though they have no clinical signs (Victorsson, 2015). A recent analysis of SVA data from the last two years (from May 2014 to May 2016) revealed a *Giardia* prevalence of 13,04% in dogs and 9,68% in cats. Once more, dogs below one year old had higher prevalence (26,33%) then adult dogs (9,8%). That trend was also observed in cats but with lower expression (11,81% in kittens and 8,73% in adult cats).

To diagnose *Giardia* infection, from dog and cat faeces, is a part of the everyday routine of

SVA Parasitological Laboratory. Therefore, it is of great importance to choose a time and cost effective diagnostic method, combined with a high sensitivity. Currently, the IF is the technique used in *Giardia* diagnosis. It is performed from one or three faeces samples (from the same animal), depending on what it's sent by the veterinary or pet owner. As there is no recommendation on the number of samples to be used in IF, a study should be performed to evaluate how many samples would return a higher sensitivity. As the IF technique sensitivity was never determined internally in SVAPL, some doubts about its accuracy have also been raised (SVA, 2016).

7. Diagnosis

The *Giardia* intermittent shedding pattern represents the major barrier for an accurate diagnosis. Shedding peaks can occur from two to seven days, and infected dogs can shed between 26 and 114,486 cysts per gram of faeces, while cats may be undetectable or shed up to 1000000 cysts per gram of feces (Tangtrongsup & Scorza, 2010). Therefore, it is recommended the examination of faecal samples from three different days over a ten days period even though this procedure cannot assure the infection detection (Garcia & Shimizu, 1997).

There is no *gold standard* test for *Giardia* detection (Gotfred-Rasmussen, Lund, Enemark, Erlandsen, & Petersen, 2016) so different laboratory methods can be used, like microscopy to detect the cyst or the trophozoite stage, enzyme-linked immunosorbent assay (ELISA) to detect parasite antigen in faeces, immunofluorescence to detect both trophozoites and cysts in faeces (Conboy, 1997), immunochromatographic kits and also polymerase chain reaction (PCR) (Baneth et al., 2015). Recently, it was described a new diagnosis method based on the volatile organic compound (VOC) profiling, which result from the unique metabolic pathways from *Giardia* due to the absence of mitochondria (Bond et al., 2015). Flow cytometry (FC) advantages will be addressed later on, as well as Mini-Flotac® which, despite its increasing use at laboratory level, still needs to be improved for protozoa diagnosis, which might play a future role in *Giardia* diagnosis.

Usually, the detection of *Giardia* and *Cryptosporidium* in clinical samples is based on an initial faecal flotation to concentrate cysts and oocysts, followed by microscopic examination of a direct faecal smear or stained, by acid-fast if cryptosporidiosis or Lugol iodine if giardiosis (Coklin et al., 2011).

As samples, it's possible to use duodenal fluid, biopsy and jejunal impression smear, but faeces are the most commonly used (Rajurkar, 2012).

a) Microscopic Techniques

Microscopic examination of stools has been the method of choice for a long time, and most laboratories still rely on it. It is easy to perform, inexpensive and with 100% specificity. However, the sensitivity of this method is low so it requires at least three faecal examinations over the course of seven to ten days, or more than one copro-parasitological method, to be possible to detect and recognize the parasites during routine direct smear microscopy. This may be explained by the irregular and intermittent shedding or by the low numbers of cyst excretion in faeces (Conboy, 1997; Khan et al., 2014; Bond et al., 2015). It is also important to use proper techniques, have a trained and experienced technician and also to make a good recognition of morphological characteristics and know some features, like the trend for trophozoites to predominate in diarrheic faeces, while cysts tend to be passed in normally to semi-normally formed stools (Conboy, 1997).

Microscopy technique can be used in combination with faecal concentration methods, like ZSCT or a simple flotation technique, to detect the cyst stage of the parasite in faeces, or with just a direct smear of faeces to detect the trophozoite stage (Conboy, 1997).

For rural settings or emergency outbreak scenarios, low-cost and portable chip-scale microscopes, like the sub-pixel sweeping microscopy (SPSM), can be used with reliable performance (Lee et al., 2014).

a1) Faecal Direct Smear

The faecal direct smear method has shown limitations in *Giardia* diagnosis and, therefore, lower sensitivity than other methods. As trophozoites are extremely sensitive to environmental stress and don't survive for long periods after the stool has been passed, faeces should be examined within 20 min (preferably immediately) after the sample has been collected, which is not always possible. On the other hand, the procedure is simple consisting in mixing a small amount of faeces in a drop of saline on a slide followed by the application of a coverslip (Conboy, 1997).

a2) Staining methods

The observation of the cysts internal morphology details can be improved by staining the sample with Iron-hematoxylin, Trichrome (figure 8), Iodine (figure 9) or Giemsa (Rajurkar, 2012; Koehler, Jex, Haydon, Stevens, & Gasser, 2014). According to Conboy (1997), the trophozoites are easily detected if alive and motile, at a 100x magnification, so using a stain may not be beneficial therefore, since it will kill the trophozoites.

Figure 8 – *Giardia* trophozoite, dividing trophozoite and cyst stained with Trichrome (DPDx, 2013).

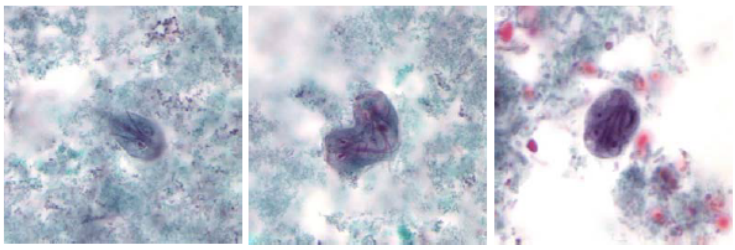
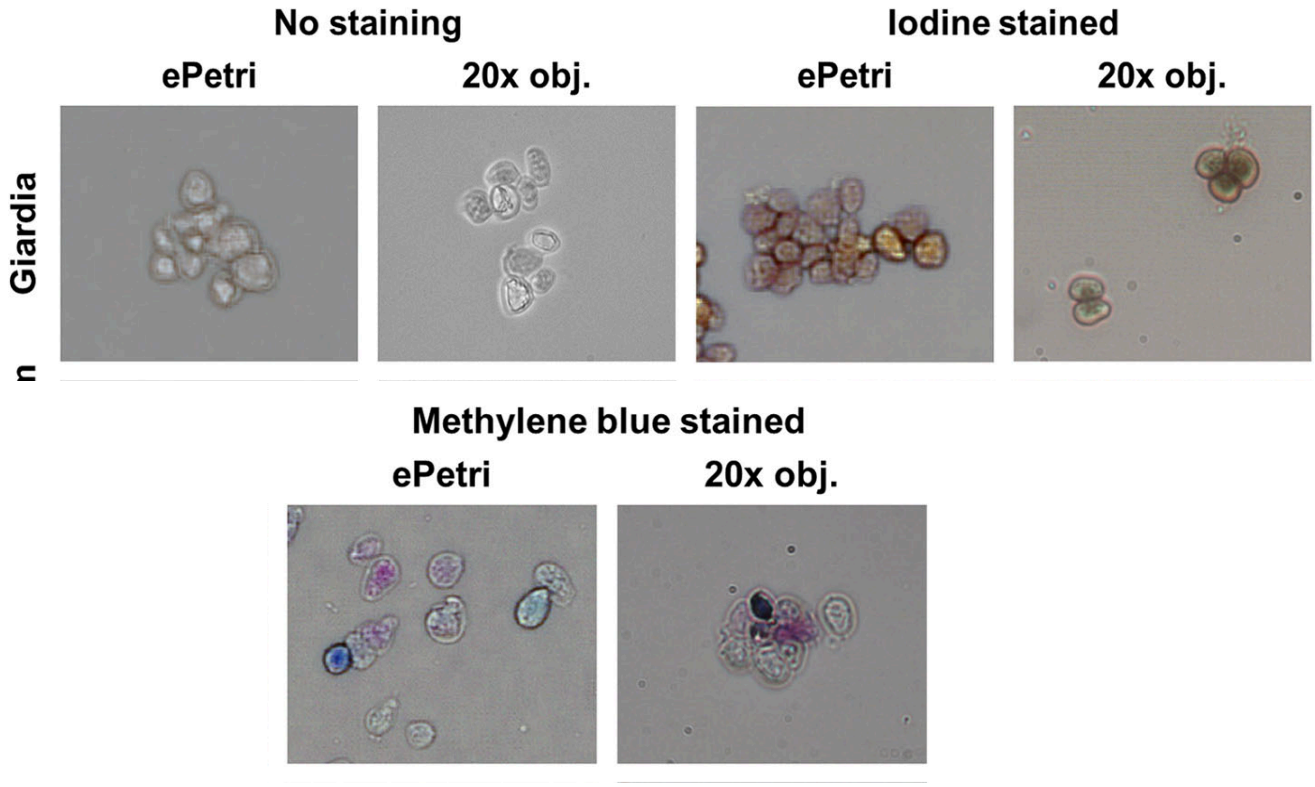


Figure 9 - SPSM and 20x objective microscope of *Giardia* cysts with different stains (Lee et al., 2014).



On the other hand, Rajurkar (2012) suggests that the methylene blue staining is the permanent staining method more suitable to preserve the trophozoite structure, since it usually rapidly disintegrates (figure 8).

a3) Faecal Flotation Methods

The faecal concentration method is the most used to examine faeces samples. It has proven to be advantageous over direct smear preparations, with 85,3% sensitivity and 99,7% specificity (when compared with immunofluorescence technique) (Khan et al., 2014; Mekaru, Marks, Felley, Chouicha, & Kass, 2007). It increases the recovery of eggs and larvae of helminths and cysts of protozoa, removes debris and enhances the visibility of the structural detail of

cysts, justifying its use as a routine diagnostic procedure. Faecal flotation is based on the density difference between the parasite products in the animal faeces and the fluid suspension medium (which has a higher specific gravity). As parasite eggs, cysts, and oocysts have lower density, they concentrate on the surface of the solution, resulting in a clean preparation for microscopic examination with a minimal amount of faecal debris (Zajac & Conboy, 2012; Bineshlal, Thiyagarajan, & Jayakumar, 2015). If associated to an immunoassay test, like SNAP® *Giardia*, its sensitivity can be increased. Although this method is easy to perform, inexpensive and allows to evaluate other parasites besides *Giardia*, it requires trained technicians, microscope, centrifuge and good laboratory procedures, as well as the use of flotation solutions (Mekaru et al., 2007). Despite the centrifugation step can be replaced by letting the mixture sit on the benchtop for a specified time, in some situations, if not performed, can substantially reduce the sensitivity of the faecal exam. It is particularly important in infections with low number of parasite forms, usual with *Giardia*, and also when using 33% $ZnSO_4$ or sugar solution. The slightly lower specific gravity (SPG) of $ZnSO_4$ solution and the high viscosity of sugar solution retard the flotation process, so the centrifugation is mandatory (Zajac & Conboy, 2012).

The chosen flotation solution has an important role in the sensitivity of these tests so it should be selected according to the parasites to be identified. The higher the SPG of the flotation solution, the greater the variety of parasite eggs that will float, but the risk of damaging the eggs from the hyperosmotic solution will increase and more debris will also float (Zajac & Conboy, 2012).

Different concentration methods have been described: saline sedimentation, formol-ethylaceate (FEA), formalin-ether sedimentation (FE, described in 1948), zinc sulfate concentration technique (ZSCT, described in 1938) and Sheather's sugar flotation technique (SSF) (Gotfred-Rasmussen et al., 2016).

Saturated solutions of sodium chloride (SPG 1.20) are widely used for being inexpensive, easy to prepare and effective in floating common helminth eggs and protozoan cysts. However, the slides must be examined relatively quickly to avoid the formation of crystals that make the observation harder (Zajac & Conboy, 2012).

For *Giardia* cysts, which collapse rapidly in most flotation solutions, the more indicated is a 33% $ZnSO_4$ solution (SPG 1.18) because it does not cause rapid destruction of *Giardia* cysts (Zajac & Conboy, 2012). Also, the ZSCT allows *Giardia* cysts to be more easily recognized in flotation preparations and has 100% specificity. However, the sensitivity is only 70% if only a single sample is analysed, increasing to 95% with the examination of three faecal specimens within five days (Papini, Carreras, Marangi, Mancianti, & Giangaspero, 2013).

Decock et al. (2003), however, suggest that two ZSCT at 24 hours and three ZSCT have the same sensitivity. This shows that *Giardia* diagnosis should not be ruled out based on a single negative faecal examination (Schuurman, Lankamp, van Belkum, Kooistra-Smid, & van Zwet, 2007). In a comparative study, FE and ZSCT were considered equally sensitive (Gotfred-Rasmussen et al., 2016).

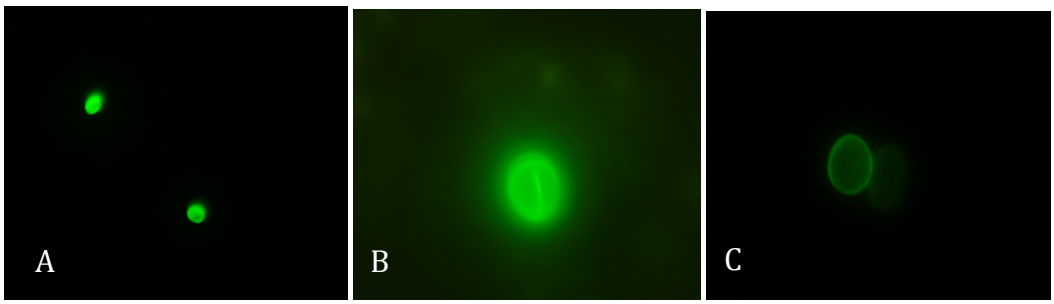
The Sheather's sugar solution (SPG 1.2–1.25), when compared with other salt solutions, is more effective for the flotation of tapeworm and nematode eggs of higher density, and it does not distort eggs so quickly. It is inexpensive, easy to prepare and it's recommended for *Cryptosporidium* oocysts recovery in faecal samples. For *Giardia* detection is less effective than 33% ZnSO4 solution (Zajac & Conboy, 2012). The SSF technique has the advantage to allow further analysis with PCR, increasing the sensitivity, although is rather laborious and not the best option in a diagnostic laboratory (Khan et al., 2014; Gotfred-Rasmussen et al., 2016).

b) Immunological methods

Immunofluorescence microscopy and the direct fluorescence antibody (DFA) test are more accurate then light microscopy, achieving high specificity (99.8–100%) and sensitivity (93–100%). The detection is based on a fluorescein isothiocyanate-conjugated anti-*Giardia* monoclonal antibody that recognises surface epitopes on cysts (Koehler et al., 2014).

Immunofluorescence microscopy can be used to detect *Giardia* cysts (figure 10) and *Cryptosporidium* oocysts in water samples and also in faecal samples from animals (Lechevallier, Norton, Siegel, & Abbaszadegan, 1995). It can be used to stain fecal smears without initial concentration but, due to the higher costs and time consuming, is generally used only in research laboratories (Gotfred-Rasmussen et al., 2016). Although more sensitive than conventional microscopy, the sensitivity can be compromised if applied in a sample with low cysts concentration (Coklin et al., 2011).

Figure 10 – Immunofluorescence images of *Giardia* cysts. A: 250x amplification. B: 800x amplification. C: 600x amplification. Original.



Using the direct immunofluorescence principle, different commercial tests were developed. **Merifluor**® *Cryptosporidium/Giardia* is an easy one-step 30 minutes single-test that uses combined fluorescein isothiocyanate-labeled monoclonal antibodies directed against the cell wall antigens of *G. lamblia* cysts and *Cryptosporidium parvum* oocysts (Zimmerman & Needham, 1995). According to the manufacturer, it is more sensitive than traditional staining methods and commercial Enzymatic Immunoassay (EIA) procedures, with 100% sensitivity and 100% specificity for *Giardia*.

The **Xpect**® *Giardia/Cryptosporidium* test is a lateral flow immunoassay for the detection of both *Giardia* and *Cryptosporidium* antigens, using antibody-coated color particles that bind with the target antigen. According to the manufacturer, it has 95,8% to 97,9% sensitivity, takes only 15 minutes and the positive results are given by a clear visible line, blue for *Giardia*, red for *Cryptosporidium*. However, when compared to direct immunofluorescence in a study, it showed lower sensitivity (79.4%) (Mekaru et al., 2007).

DFA and ELISA, as well as the molecular method to amplify DNA by PCR, are diagnostic techniques developed to diagnose canine giardiosis with only a single test. These tests have higher costs, requires sophisticated and expensive equipment as well as trained personnel. For many veterinary clinics laboratories, ELISA-based rapid diagnostic test kits represent a practical and time-saving alternative and are often used (Papini et al., 2013) .

These enzyme-linked immunoassays detects the presence of *Giardia* coproantigen, with 87–100% specificity and 63–100% sensitivity (Koehler et al., 2014). They are quick, inexpensive, employ easy techniques without any special equipment (Papini et al., 2013), and can be indicated for large-scale environmental screening programs to assess the risk of exposure to *G. duodenalis* cysts of canine source in human settlements (Papini et al., 2013). However, despite the sensitivity of these tests on a single sample is higher than that of conventional microscopy methods, in asymptomatic animals it might be necessary to analyse two samples in order to reach sensitivities above 90% (Hanson & Cartwright, 2001).

There are different commercial ELISA tests available. The **Immuno*Card*** STAT!® *Crypto/Giardia* test (Meridian Bioscience, Boxtel, The Netherlands) is an enzymatic immunoassay for the qualitative detection of *Cryptosporidium parvum* and *Giardia lamblia* antigens. It is considered the most efficient, with 100% sensitivity and specificity for *Giardia*, economical, with results in 10 minutes (Koehler et al., 2014). The *Giardia/Cryptosporidium* **Chek**® test (TechLab, Inc.), has the advantage of testing multiple specimens simultaneously, 96 on each plate, being cost and time-efficient. According to the manufacturer, the total assay time is under two hours, and it has 99,3% sensitivity and 100% specificity. It allows both

Giardia and *Cryptosporidium* antigens qualitative detection in faecal samples, but a positive result doesn't distinguish which one of the parasites is present (Youn, Kabir, Haque, & Petri, 2009). The Idexx **SNAP**® *Giardia* test has lower sensitivity then Immuno*Card* STAT!® (89,2% according to the manufacturer) and its use has been recommend in combination with flotation techniques to optimize the cysts detection. The **ProSpecT**® *Giardia* Microplate Assay EIA is another available test, with 91.2% sensitivity and 99.4% specificity (Mekaru et al., 2007).

c) Molecular Methods

The **real-time PCR** assay measures *Giardia* cyst DNA in faecal samples (Papini et al., 2013). It is used to amplify fragments of various genes to enable the detection of parasite DNA (Coklin et al., 2011). When compared to IF as the reference method, the qPCR exhibits 91% sensitivity and 95,1% specificity (Gotfred-Rasmussen et al., 2016). Like microscopy, PCR may have limited sensitivity in samples containing small numbers of cysts, largely because of the presence of PCR inhibitors. One option to improve sensitivity of PCR is by using immunomagnetic separation (IMS) as a second concentration step after flotation. It will not only concentrate cysts, but also allow for more target DNA to be extracted and PCR inhibitors to be removed (such as bilirubin, bile salts, and complex polysaccharides) from faecal debris, avoiding interference in the parasite detection (Coklin et al., 2011).

PCR gives more consistent and reliable results because the level of expertise and fatigue are not major factors. PCR is also rapid and effective, allowing the analysis of many more samples in a day than does microscopy. IMS-PCR assay has proven to be more sensitive than immunofluorescence microscopy. However, if a large number of cysts and oocysts are present, both methods can be equally used (Coklin et al., 2011).

In order to improve *Giardia* detection, ELISA and real-time PCR assay can be used simultaneously, with parallel test interpretation to increase sensitivity, or with serial interpretation to improve specificity. This can be useful to identify the risk factors associated with community outbreaks of giardiosis, even if the high costs of reagents and equipment makes it more expensive. To analyse dog stools in the environment, parallel interpretation might be the best option, since false positives are unlikely to have serious consequences (Papini et al., 2013).

d) Immunochromatography (IC)

Speed® Giardia™ (Virbac) is a quick and easy test, used to detect *Giardia* cysts antigens in dogs, cats and cattle, in only 10 minutes. When compared with the reference method laboratory ELISA, it shows 95.6% sensitivity and 100% specificity, with a detection threshold of 80 cysts per gram of faeces. IC tests have the advantage of detecting the infection prior to the excretion of the cysts in host faeces, and can be a low-cost choice for screening large numbers of faecal samples. However, the cross-reactivity (affecting specificity), the intermittent shedding of cysts and *Giardia* antigens, the use of formalin as a fixative (which reduces sensitivity) and the low numbers of cysts in faeces or trophozoites in the host intestine, can decrease the sensitivity and specificity to values lower than microscopy techniques. Some IC kits can also lead to subjective interpretations because results are based on staining intensity and interpretation (Koehler et al., 2014).

e) Flow Cytometry (FC)

Flow cytometry (FC) is a quick and sensitive method for screening large numbers of faecal samples for the presence of protozoan cysts and oocysts in a shorter time with consistent results. It is not confirmatory itself, so positive or equivocal samples should be readily examined and confirmed by IF. FC can be an effective alternative method for the detection of *G. duodenalis* cysts, especially for large scale epidemiological studies or extensive surveillance programs, however it's not as sensitive as IF (Uehlinger et al., 2008).

f) Mini-FLOTAC®

The Mini-FLOTAC® device is a new method for the diagnosis of helminth infections (B. D. Barda et al., 2013) and protozoa, like *Eimeria* spp. (Silva et al., 2013). It is based on eggs flotation and combines sensitivity and low costs (B. Barda, Cajal, et al., 2014). The procedure does not require any centrifugation step or expensive equipment, it can be performed on both fresh and fixed stool samples, and only requires 10–12 minutes of preparation before microscopic analysis (B. Barda, Ianniello, et al., 2014). These features make it a good alternative to the FLOTAC® technique, the *gold standard*, in those laboratories where the centrifugation step cannot be performed (Silva et al., 2013).

The Mini-FLOTAC® comprises two physical components, the base and the reading disc. The base has two flotation chambers (1 mL each) surmounted by a reading disc, designed for optimal examination of faecal sample suspensions (total volume = 2 mL) with a maximum magnification of 400×. To fill the chambers it can be used the Fill-FLOTAC®, a plastic device with a container, a collector and a filter, which homogenizes, filters and pours the sample in

the flotation chamber. Before observation, a 10 minutes rest is needed to allow eggs to float (Silva et al., 2013; B. Barda, Cajal, et al., 2014. As flotation solutions can be used saturated sodium chloride (density = 1.20), recommended for the diagnosis of soil-transmitted helminths, and zinc sulphate (density = 1.35), recommended for *Schistosoma mansoni* and for intestinal protozoa. The analytic sensitivity is 10 eggs/gram for sodium cloridium and 12.5 eggs/gram for zinc sulfate (B. Barda, Cajal, et al., 2014; B. Barda, Ianniello, et al., 2014).

According to Barda et.al (2013), mini-FLOTAC® is the most sensitive method for helminth infections (90%) in comparison with formol-ether concentration method (FECM) (60%) and direct fecal smear (30%), whereas FECM is the most sensitive for intestinal protozoa infections (88%) against 70% direct fecal smear and 68% mini-FLOTAC® (B. Barda, Ianniello, et al., 2014).

There are no studies exploring and evaluating the efficiency of Mini-FLOTAC® in *Giardia* diagnosis.

8. Treatment

Giardia is a zoonotic parasite that can be transmitted by house pets, namely dogs and cats, which enhances the importance of an accurate diagnosis and effective treatment. The goal of the treatment is to stop the diarrhoea and eliminate the infection.

According to ESCCAP (2011), metronidazole, tinidazole, fenbendazole and the combination of febantel/pyrantel/praziquantel are good treatment options. Other studies refer also oxfendazole and albendazole as effective, when used at the anthelmintic dosage (Decock, Cadiergues, Larcher, Vermot, & Franc, 2003), as well as furazolidone, ipronidazole and quinacrine (Decock et al., 2003).

The most commonly used drug is metronidazole, even though it's not licensed for veterinary use (Decock et al., 2003). Metronidazole is an antibacterial and antiprotozoal agent, used in *Giardia* therapy for both dogs and cats, in a dose of 25 mg/kg twice a day for five days (European Scientific Counsel Companion Animal Parasites, 2011; Plumb, 2011). It is considered the first choice when there is also a bacterial infection, like *Clostridium perfringens*, due to its antibacterial properties (Tangtrongsup & Scorza, 2010). In dogs, it was described with a treatment success rate of approximately 67% (Anderson et al., 2004). This drug is activated inside the parasite by the reduction of the nitro group and binds covalently to DNA molecules, resulting in irreversible helical damage and death of the organism (Da Silva et al., 2011). However, resistance to metronidazole is common and side effects as anorexia, vomiting and central nervous system toxicity has been described (Montoya, Dado, Mateo, Espinosa, & Miró, 2008). These secondary effects can be decreased with the addition of

silymarin as a treatment supplement to metronizadole (Chon & Kim, 2005).

Fenbendazole (Panacur®, MSD) is an antiparasitic drug, registered for the treatment of giardiosis in dogs in most European countries and also recommended for cats. Can be used at a dose of 50 mg/kg once a day for three or five days, and the treatment can be repeated if clinical signs and cyst excretions persist (European Scientific Counsel Companion Animal Parasites, 2011). As all benzimidazole antiparasitic agents, it disrupts intracellular microtubular transport systems by binding selectively and damaging tubulin, inhibiting microtubule formation, disrupts metabolic pathways within the helminth and inhibits metabolic enzymes of the parasite (Plumb, 2011). Can also be used in a combination tablet containing febantel, pyrantel and praziquantel (Drontal® Flavour Plus, Bayer) repeated once a day for three days. This treatment is licensed in some European countries and countries outside the EU (European Scientific Counsel Companion Animal Parasites, 2011) and it was proven to be highly efficacious against *Giardia* infection in dogs by Montoya et al. (2008).

Albendazole should not be considered as a treatment option due to the possibility of causing bone marrow suppression (Montoya et al., 2008).

Ronidazole, the drug of choice against *Tritrichomonas foetus* in cats, in combination with the disinfection of the environment and shampooing of the dogs, has proven to be highly effective in reducing *Giardia* cyst excretion, representing a treatment option to canine giardiosis (Fiechter et al., 2012).

Secnidazole, a human medicine drug, has been studied as an alternative drug for cats, having demonstrated a curative efficacy of 100% in cats infected by *G. duodenalis.* It has the advantage to be administered in a single dose, but it still needs to be studied, approved and registered for veterinary usage (Da Silva et al., 2011).

Vaccination protocols were also tested in asymptomatic dogs treatment, but they have proven to be ineffective (Anderson et al., 2004).

The use of probiotics, which are beneficial in diarrhoea therapy, do not decrease *Giardia* infection rates when used alone (Bybee, Scorza, & Lappin, 2011).

9. Prevention of Infection

Even though *Giardia* treatments in dogs and cats have proven to be very effective, treatment failure may occur due to re-infections, co-infections, other underlying disease or by incomplete parasite removal following treatment (European Scientific Counsel Companion Animal Parasites, 2011). To prevent it, hygienic measures must be associated to avoid recontamination (Decock et al., 2003). The prompt removal of faecal material, preventing dogs from consuming contaminated surface water or faeces, and the disinfection and cleaning

of kennels, are mandatory measures. This can be accomplished with 1% sodium hypochlorite (20% commercial bleach), 2% glutaraldehyde or quaternary ammonium compounds. Cysts are relatively resistant to chlorination, so levels of chlorine in drinking water are inadequate to inactivate them, but, as being susceptible to desiccation, cleaning and thorough drying will kill them (Esch & Petersen, 2013). When treated with antiprotozoal drugs, the use of a shampoo containing chlorhexidine digluconate, to bathe the treated animal at the beginning and at the end of the treatment, may also be useful in reducing re-infections (European Scientific Counsel Companion Animal Parasites, 2011).

In humans, it is critical to ensure a good fresh water provision, adequate sewage systems, fresh food properly cleaned and personal hygiene habits reinforced. Faeces from infected animals should be frequently removed followed by proper disinfection (Baneth et al., 2015).

IV – RESEARCH STUDY: "Evaluation of faecal sampling methods for the analysis of *Giardia* sp. in companion animals"

1. Objectives

This study aims to analyse the difference of IF technique accuracy between using three faeces samples and only one sample, from dogs and cats, and to determine the relative sensitivity and specificity. A prevalence analysis was also accomplished to evaluate if there were significant differences between the number of positives identified with one sample and with three samples.

It also aims to evaluate how the technique used to collect a faecal subsample with the swab can influence the method sensitivity: if only one scratch is representative or if more scratches should be performed in different areas of the faecal sample to increase the chances of finding the cysts. This comparison wasn't found in any research article, which increases and justifies the importance of its outcome.

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2. Material and Methods

a) Immunofluorescence Microscopy

The chosen protocol for this study is a modification of the one used by SVA Parasitology Laboratory, and was adjusted according to the study goals in what concerns the subsampling procedure (figure 12).

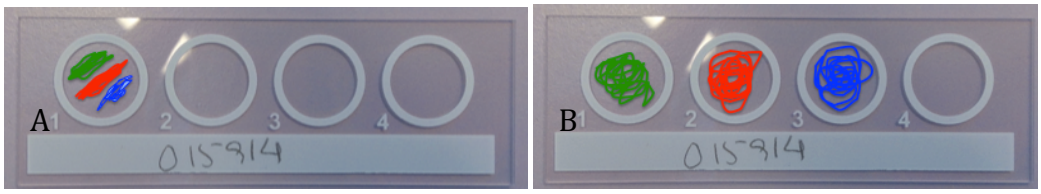
Both require a teflon printed diagnostic slide with four wells with 11mm (figure 11). A monoclonal antibodies solution (A100 FLR: Aqua-G/C, Waterborne Inc) was previously prepared.

Figure 11 – Teflon printed diagnostic slide with 4 wells with 11mm. Original.

Faeces from 272 animals (239 dogs and 33 cats) were collected in three different moments and sent to SVA Parasitology Laboratory (SVAPL) for *Giardia* diagnosis. They were tested according to the routine IF method used in SVA, and also according to the study IF protocol. For the prevalence analysis, the SVAPL data from 433 animals with only one sample was used, as well as the data from 254 samples from the group with 3 samples.

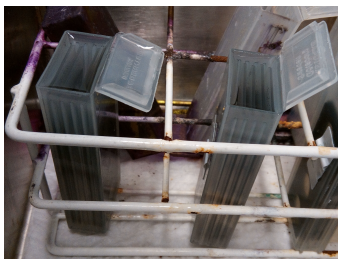
The only difference between SVAPL and the study method was how the faeces were placed in the slide. SVAPL created a pool of the three samples in one well of the slide while the study method placed each one of the samples in a different well. In figure 12, each color represents a different sample.

Figure 12- A: SVAPL pool of three samples mixed in well number 1; B: Study slide with each sample separated by three different wells (1, 2 and 3). Each color represents a different sample from the same animal. Original.



To prepare the slide, a swab was used to make a single scratch in each of the three samples and applied separately on the slide on the first three wells. On the fourth well, a second analysis from the sample of the third well was made, to test the swab subsampling step of the IF technique. The collection of a small amount of faeces with the swab to be placed in the slide was different between the SVAPL and the study methodology. If performed according to SVAPL, only one place of the faeces was used (single scratch), whereas according to the study, different places from the same sample were scratched (multi-scratch). The swab was changed between samples to avoid contaminations. After 30 minutes drying, the sample was fixated in methanol for 5 minutes (figure 13), and dried again for at least 10 minutes.

Figure 13 - Samples fixation in Methanol. Original.



Afterwards, 25µl of monoclonal antibodies solution was applied in each well and the slide was incubated 30 minutes at 37ºC. Then, it was washed and immersed in PBS (0,01M, pH 7,2) solution for 2 minutes and dried again for 10 to 20 minutes. A drop of glycerol with 0,1M $NaHCO_3$ solution was applied in each well, and the teflon slide was covered with a 24x60mm cover slip.

The slide was then ready to be examined in the immunofluorescence microscope with a 495nm filter. A 25x objective was used, as well as a drop of immersion oil for each well. The samples were examined for the presence of *Giardia* and the number of cysts was counted in each well.

b) Statistical analysis

The statistical analysis was performed in Epitools® (AusVet Animal Health Services (AVAHS), 2016), using different tests. The McNemar's test, a modification of the chi-squared test, was applied by a 2x2 contingency table to assess the significance of the difference between the two correlated proportions. As there is no *gold standard* for *Giardia* diagnosis, it became necessary to evaluate the agreement between the two methods (SVAPL and study) without assuming that one is better than the other. To measure the proportion of agreement beyond that to be expected by chance, was calculated the *kappa* value. The statistic ranges from 0 to 1 with a kappa value of 0 indicating poor agreement, 0 to 0.20 slight agreement, 0.21to 0.40 fair agreement, 0.41 to 0.6 moderate agreement, 0,61 to 0,8 substantial agreement and 0,81 to 1 almost perfect agreement (Thrusfield, 2007).

As the objective of IF is to rule out disease, a reliable negative result is required and therefore the test should have high sensitivity and generate few false negatives. Because there is no *gold standard* for *Giardia* diagnosis, a relative sensitivity and specificity were determined by using a 2-by-2 table layout and the appropriate formulas.

A two-sample test of proportions, using Epitools® (AVAHS, 2016), was used to see if the apparent prevalence of two different groups, one with one sample per animal and one with three samples per animal, differs significantly, and the confidence limits were calculated using Wilson Score test. These are apparent prevalence because are based on the number of animals tested positive in the diagnostic test, not considering the sensibility.

3. Results

A total of 272 animals (239 dogs and 33 cats), most with diarrhoea or clinical signs of gastrointestinal disease, were tested for *Giardia* infection by IF method (total results in table 8 in appendix 1). According to the study, 44 animals were true positives (TP) and 222 were true negatives (TN), with 6 false negatives (FN). From each animal, 3 samples were analysed and grouped randomly in 3 groups, so that each group had one of the three samples from each animal. A statistical comparison was performed between each one of the groups and the SVAPL diagnosis, based on the 3 samples pool analysis, using McNemar's and *kappa* test. It was also compared the SVAPL results with the study results, considering the SVAPL method

as the *gold standard*. In Table 1, it is possible to see the outcome from that analysis. The TP, TN, FN and percentage of missed positives (table 2) were calculated for each group by comparison with the result in parallel of the three diagnostic approaches (groups).

Table 1 – Statistical comparison analysis between each group and the SVAPL diagnosis results, and between SVAPL and the study results, using McNemar's test and *kappa* test.

		McNemar's test	
	kappa	p (Chi sq)*	Overall proportion agreement
Samples day 1 vs SVAPL	0,8225	0	96%
Samples day 2 vs SVAPL	0,8919	0,02	97%
Samples day 3 vs SVAPL	0,8751	0,01	97%
SVAPL vs study	0,7909	0,61	95%

* Statistically significant for $p < 0.05$

Table 2 - Results from the analysis of the single samples grouped in 3 groups randomly and from the SVAPL method, in comparison with the study results: TP, TN, FN and missed positives percentage.

	TP	TN	FN	% Missed positives
Samples day 1 vs Study	30	228	14	32
Samples day 2 vs Study	34	228	10	23
Samples day 3 vs Study	33	228	11	25
SVAPL vs study	41	222	9	20

Data in table 8 in appendix 1, and not considering the SVAPL method as a *gold standard*, shows nine false negatives for the SVAPL method detected by the study method. The number of cysts identified in each well can be seen in Table 3. The six false negatives from the study method were not included in table 3 because in SVAPL diagnosis the number of cysts is not counted, so they can't be used to evaluate the concentration of cysts in false negatives samples.

Table 3 - Number of cysts found in each well from the slides representing the SVAPL false negatives samples that were positives according to the study method ("neg" = negative, "+" = positive).

Sp	ID	sample1	sample2	sample3	Multi	SVAPL	Study
dog	14796	1	0	0	0	neg	+
dog	15891	0	2	1	0	neg	+
dog	16244	0	1	0	0	neg	+
dog	18057	1	0	0	0	neg	+
dog	19592	0	28	4	5	neg	+
dog	21851	0	1	0	1	neg	+
dog	23808	0	0	1	0	neg	+
dog	23775	1	1	0	1	neg	+
dog	25533	1	0	0	0	neg	+

To calculate the prevalence of giardiosis in dogs and cats tested in SVA, samples sent since 11th of April till 20th of May 2016 were analysed. From a total of 687 test requests, 433 had 1 stool sample and 254 had 3 stool samples. The number of TP, TN and FN were determined for the total of animals. Dogs and cats were also analysed separately, each considering the total number of animals, animals younger than one year old and animals older than 1 year old. For the group with one sample, was not possible to determine the FN because only the SVAPL tested the samples. The results are presented in Table 4.

Table 4 – Number of TP, TN and FN from the faeces analysis of dogs and cats, for both animals with one and three samples.

	Animals with 1 sample			Animals with 3 samples			
	Total	TP	TN	Total	TP	TN	FN
Total (dog+cat)	433	52	381	254	35	215	4
< 1 year	117	25	92	62	14	46	2
> 1 year	301	23	278	186	21	164	1
Dog	246	36	210	223*[1]	31	190	2*[2]
< 1 year	56	18	38	52	14	38	0
> 1 year	182	15	167	165	17	147	1
Cat	187	16	171	31	4	25	2
< 1 year	61	7	54	10	0	8	2
> 1 year	119	8	111	21	4	17	0

*[1] 6 animals didn't have information about the age
*[2] 1 FN didn't have information about the age

In table 5, are presented the apparent prevalence calculated from table 4 data, as well as the apparent prevalence from all the samples tested in SVA over the past 2 years. A two-sample test of proportions was used to evaluate if there is any significant difference between prevalence from the groups with 1 sample and the groups with 3 samples. Confidence intervals were calculated using Wilson Score Interval.

The study IF method relative sensitivity and specificity was determined for dogs, cats and total (table 6), using the 272 samples from table 8 in appendix 1. In what concerns to the specificity, since there were no false positives, or at least it was not possible to distinguish between a true positive or a possible contamination, it was considered 100%.

Table 5 – Apparent prevalence of giardiosis from dog and cat faeces analysed in SVA over the last 2 years, and also from samples sent from 11th April till 20th May 2016, grouped considering the number of samples sent from each animal (one or three samples). Prevalence from one sample group and three samples group were compared, using two-sample test of proportions (p-value), and confidence interval were found using Wilson Score.

	SVA 2 years	1 sample		3 samples		
	Prevalence (%)	Prevalence (%)	95% CI	Prevalence (%)	95% CI	p*
Total (dog+cat)	11,69	12	[9.3, 15.4]	15,35	[10.1, 18.6]	0,5
Dog	13,06	14,63	[10.8, 19.6]	14,80	[10, 19.1]	0,8
< 1 year	26,33	32,14	[21.4, 45.2]	26,92	[16.8, 40.3]	0,5
> 1 year	9,8	8,24	[5.1, 11.2]	10,98	[6.5, 15.9]	0,5
Cat	9,68	8,56	[5.3, 13.4]	19,35	[5.1, 28.9]	0,4
< 1 year	11,81	11,48	[5.7, 21.8]	20	[0, 27.8]	0,27
> 1 year	8,73	6,72	[3.4, 12.7]	19,05	[7.7, 40]	0,06

* Statistically significant for $p < 0.05$

Table 6 - Study IF method relative sensitivity and specificity for dogs and cats as a group, and for both as two separated groups.

	Total (dogs and cats)	Dogs	Cats
Sensitivity	88%	91%	71%
Specificity*	100%	100%	100%

* The specificity was considered 100% due to the impossibility to identify the FP.

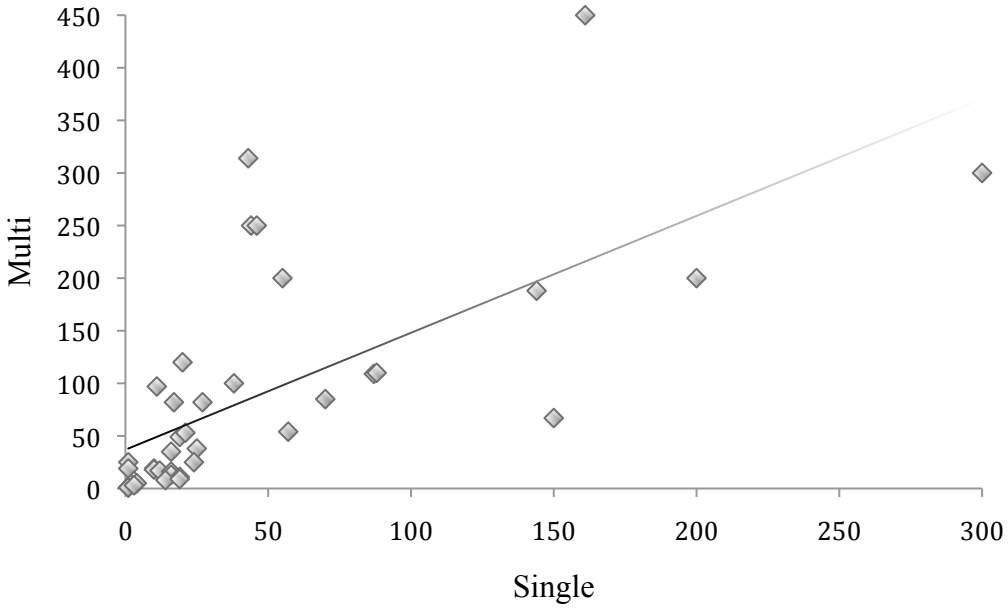
To evaluate the subsampling of the faeces with the swab, 282 samples were analysed, from which 228 animals were diagnosed as negatives and 54 as positives (considering the study diagnosis). For the negative animals, both SVAPL and study were negative. For the 54 positives, 43 were identified using a single scratch (missed 11 positives, of which five were detected by multi-scratch technique), and 44 were identified using multi-scratch (missed 10 positives, of which 4 were detected by single scratch technique). Complete table with all data is presented in table 9 in appendix 2.

In table 7 are represented the number of cysts found in each positive sample, and the ratio between single and multi-scratch technique. The relation between the number of cysts found in both techniques, for the same animal, is represented in graphic 1. Graphic 2 shows the average amount of cysts found in both single and multi-scrach techniques, as well as the average ratio given by these two techniques relation.

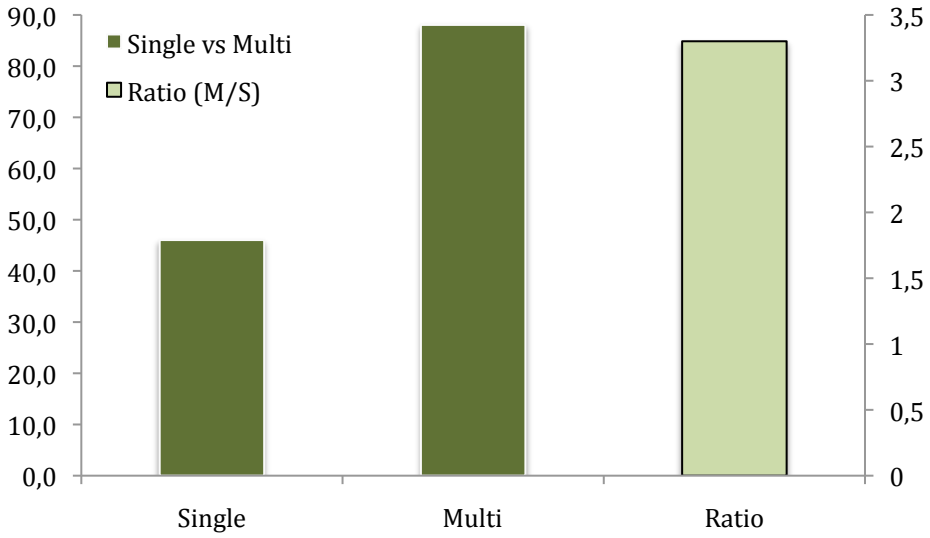
Table 7 – Number of *Giardia* cysts counted using single and multi-scratch technique, in positive samples for both techniques. The ratio (Multi/Single) between the two results was calculated for each animal.

Animal	ID	Single	Multi	Ratio (M/S)
dog	18342	1	25	25,00
cat	24577	1	19	19,00
dog	14774	11	97	8,82
cat	22284	43	314	7,30
dog	18809	20	120	6,00
dog	15289	44	250	5,68
cat	14030	46	250	5,43
dog	15583	17	82	4,82
dog	25889	55	200	3,64
dog	25892	27	82	3,04
dog	15281	161	450	2,80
dog	25916b	38	100	2,63
dog	25526	19	49	2,58
dog	22769	21	53	2,52
dog	17458	3	7	2,33
dog	22763	16	35	2,19
dog	19516	2	4	2,00
dog	14289	10	19	1,90
dog	24627	10	18	1,80
dog	25568	25	38	1,52
cat	25109	12	17	1,42
cat	22282	144	188	1,31
cat	16935	87	109	1,25
dog	19592	4	5	1,25
dog	21511	88	110	1,25
dog	25916a	70	85	1,21
dog	18792	24	25	1,04
dog	14020	1	1	1,00
dog	15679	1	1	1,00
dog	17177	16	16	1,00
dog	18804	3	3	1,00
dog	25920	300	300	1,00
dog	26775	200	200	1,00
dog	17182	57	54	0,95
cat	25551	16	13	0,81
dog	24991	19	11	0,58
dog	25569	14	8	0,57
dog	23285	19	9	0,47
dog	26449	150	67	0,45

Graphic 1 – Relation between the number of cysts counted using single and multi-scratch technique, in positive samples for both techniques.



Graphic 2 – Average data of single and multi-sampling, and respective ratio.



4. Discussion

Considering table 1 and table 2, we realize that there are significant differences between using one sample and three samples to diagnose *Giardia*. By using only one sample, a relevant percentage of positives was missed, so the use of three samples should be adviced as it is for other techniques, such as conventional microscopy, ZSCT and ELISA (Conboy, 1997; Hanson & Cartwright, 2001; Papini et.al, 2013). The almost perfect agreement, given by *kappa*, between each comparison group seems to be controversial to the p-value interpretation

but it only shows that most of the results are in agreement, which doesn't mean that the ones that aren't have no significance. These results might be justified by the low prevalence of giardiosis.

There were no differences between the SVAPL and the study results, even though SVAPL had 9 false negatives and the study 6 false negatives. However, the number of cysts found in the positive samples identified by the study, that turned out to be false negatives for the SVAPL, was very low (only 1 or 2 cysts per well) in most of them. This is in accordance with Coklin et al. (2011) statement about the low IF sensitivity in faeces with low cysts concentration, explaining the false negatives in both methods. There are some theories that could also explain the false negatives, besides the low cysts concentration. It could been due to a contamination (leading to a false positive), to a bad technique execution (insufficient sample drying before the fixation, a problem with the monoclonal antibodies or the small sample amount used in the slide) or simply due to the wrong place of the faeces used to collect the small amount to test. As the number of stool samples with low cysts concentration was substantial, it leads to a pertinent question: how relevant is that small number of cysts for the animal clinical condition? Is *Giardia* the true responsible for the clinical signs or was just an insignificant finding? This highlights the need of a close cooperation betweeen the veterinarians and the laboratories, so that it can be discussed the real importance of the diagnosis result and the eventual need for further tests. However, we should always bear in mind that, in case of a zoonotic assemblage, animals whose samples have low cyst concentration should be treated and properly monitored. These results show that, when using three samples, it is not relevant if they are tested in a pool or separately, which is important for the SVAPL when it comes to decide in which way the technique should be executed, since it wasn't found any study focused on this matter.

IF is indeed a high sensitivity technique but, as seen before, it is compromised when faeces with low cysts concentration are tested. This suggests that further studies should be developed in order to understand in which way this gap could be eliminated, considering what is possible and affordable to be used as a routine diagnosis method in SVAPL. One option could be the addition of a concentration step to increase the amount of cysts in the sample, even though it increases the dilution factor. Other option includes the concomitant use of a snap test, with the disadvantage of increasing the costs for the animal owners. Either way, it would be important to evaluate with ZSCT the negatives samples (previously tested with IF) to clarify if the number of false negatives can be even higher.

Giardiosis prevalence given in table 5 shows that dogs (around 15% for both one sample and 3 samples group) and cats (8,56% and 19,35%, for one and 3 samples group, respectively)

prevalence were within the normal range described in previous studies by Zanzani, Gazzonis, et al. (2014), also with higher prevalences for puppies and kittens. Prevalence between animals with one sample and three samples were very similar, even though slightly higher for the three samples group. However, in the cat group, the prevalence from animals with 3 samples were much higher that the prevalence from animals with 1 sample. This might be explained by the low number of animals tested with three samples (31) in comparison with the number of animals tested with only one (187).

Prevalences between one sample and three samples group were not significantly different and both are in accordance to the prevalence from the animals analysed in SVA during the last two years (with exception for the cats from the three samples group) (SVA, 2016).

These results could suggest that there is no difference in using one or three samples to diagnose *Giardia* in companion animals. However, the tested samples in the last two years and the ones from the one sample group were only analysed by the SVAPL method, so it was not possible to identify any false negatives, which would influence the final results.

The relative sensitivity and specificity of the test were also assessed (table 6). The sensitivity values are below the ones described by Koehler et al. (2014) for IF (93-100%) but only for the cats group the difference is really marked. It can be explained by the small number of animals (31 cats versus 223 dogs) that were tested for *Giardia*, and also by the high number of samples with a low cyst concentration, that decreases the method sensitivity, as mentioned by Coklin et al. (2011). The IF sensitivity achieved with this research study has proven to be higher than the ZSCT 71% sensitivity value described by Papini et.al (2013) studies, obtained with one sample tested. However, if 3 samples are analysed using ZSCT, the sensitivity (95%) will be higher than the sensitivity value from this study. Since this method concentrates the cysts, and as long as centrifugation step is assured (Zajac & Conboy, 2012), it can be a good alternative for *Giardia* diagnose instead of IF, or it can be an additional step to the IF procedure itself, in order to improve IF sensitivity.

Other methods, like qPCR and IC, studied by Gotfred-Rasmussen et al. (2016) and Koehler et.al (2014), despite apparently having a slightly higher sensitivity (91% and 95,6%) then IF, have disadvantages which invalidates them as a routine diagnosis test for *Giardia* infection. According to these autors, both tests sensitivity are compromised by samples with low cyst concentration, as it is for IF technique, so they add no value concerning to this matter. They also refer that the cross reactivity and use of formaline as a fixative in IC also decrease the sensitivity and specificity of this test so, despite it detects the infection prior to the cyst excretion and represents a low cost option for screening a large number of samples, the results are not reliable so it cannot be considered a good alternative to IF. As for IMS-PCR, despite

Coklin et.al (2011) describes it as rapid, reliable and effective test, the high costs associated exclude this technique as a routine diagnosis method.

The specificity value is in accordance to what it is described for IF method by Koehler et al. (2014), but higher then ZSCT (95%) and qPCR (95,1%) specificity, which might be explained by the impossibility of this research study in detecting the false positives.

Regarding the IF swab procedure changes, both single and multi-scratch led to false negatives (11 and 10, respectively) and both detected positive samples that weren´t detected by the other. This means that using a multi-scratch technique does not improve the sensitivity of the test, because it didn't improve the cyst detection in comparison with the single scratch technique. However, if we analyse table 7 and graphics 1 and 2, the number of cysts in the slide is higher when using the multi-scratch, which can ease the cyst detection and make the diagnosis faster and easier.

5. Conclusions

In Sweden, *Giardia* diagnosis in companion animals is an important part of SVA Parasitology Laboratory daily routine. Having IF as the main diagnostic method, it was important to test if there was a way of improving it, either by reducing the number of samples per animal, or by small changes in the procedure protocol.

This study, has once more shown that three samples per animal from different days should continue to be tested to improve IF sensitivity, due to the cysts intermittent excretion. IF has proven to be a good diagnostic technique but the accuracy is compromised when it is a sample with low cysts concentration, aggravated by the small amount used in the slide, leading to false negatives. In this way, it is important a close cooperation between the Laboratory and the small animal practitioners, so that a better clinical evaluation of the animal can be done by relating the clinical signs with the diagnosis.

Regarding the IF protocol, it is advisable to do a faeces swab from different places to increase the number of cysts and, therefore, ease the slides observation. Additional steps to the IF method, as a concentration step, should also be considered as an asset to increase the SVAPL diagnosis accuracy.

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Appendix I – *Giardia* diagnosis results from IF method.

Table 8 - General results for *Giardia* diagnosis, using the Study IF method ("+" - positive; "neg" - negative) and number of cysts found in each group. SVAPL diagnosis results for comparison. N=272 animals

Sp	ID	Group 1	Group 2	Group3	Multi	SVAPL	STUDY
dog	14751	0	0	0	0	neg	neg
dog	14796	1	0	0	0	neg	+
dog	14774	112	103	11	97	+	+
dog	14628	1	2	0	1	+	+
dog	14289	2	4	10	19	+	+
dog	14020	3	1	1	1	+	+
cat	14030	17	48	46	>250	+	+
dog	15019	0	0	0	0	+	neg
dog	15020	0	0	0	0	neg	neg
dog	15021	0	0	0	0	+	neg
dog	15023	0	0	0	0	neg	neg
dog	15289	35	> 300	44	250	+	+
dog	15281	138	109	161	>450	+	+
dog	15292	0	0	0	0	neg	neg
dog	15284	0	0	0	0	neg	neg
dog	15583	18	24	17	82	+	+
cat	15599	0	0	0	0	neg	neg
dog	15601	0	0	0	0	neg	neg
cat	15725	0	0	0	0	+	neg
cat	15724	0	0	0	0	+	neg
dog	15678	0	0	0	0	neg	neg
dog	15679	0	0	1	1	+	+
dog	15736	0	0	0	0	neg	neg
dog	15737	0	0	0	0	neg	neg
dog	15738	0	0	0	0	neg	neg
dog	15763	0	0	0	0	neg	neg
dog	15764	0	0	0	0	neg	neg
dog	15767	0	0	0	0	neg	neg
dog	15875	0	0	0	0	neg	neg
cat	15877	0	0	0	0	neg	neg
dog	15891	0	2	1	0	neg	+
dog	15893	0	0	0	0	neg	neg
dog	15914	0	0	0	0	neg	neg
dog	15912	0	0	0	0	neg	neg
dog	15911	0	0	0	0	neg	neg
dog	15917	0	0	0	0	neg	neg
dog	15941	0	0	0	0	neg	neg
dog	15942	0	0	0	0	neg	neg
dog	15958	0	0	0	0	neg	neg
dog	15935	0	0	0	0	neg	neg
dog	16244	0	1	0	0	neg	+
dog	16250	0	0	0	0	neg	neg
dog	16278	0	0	0	0	neg	neg
dog	16280	0	0	0	0	neg	neg

Table 8 (continuation) - General results for *Giardia* diagnosis, using the Study IF method ("+" - positive; "neg" - negative) and number of cysts found in each group. SVAPL diagnosis results for comparison. N=272 animals

dog	16299	0	0	0	0	neg	neg
cat	16239	0	0	0	0	neg	neg
cat	16251	0	0	0	0	neg	neg
cat	16236	0	0	0	0	neg	neg
dog	16358	0	0	0	0	neg	neg
dog	16364	0	0	0	0	neg	neg
cat	16931	0	0	0	0	neg	neg
cat	16935	105	7	87	109	+	+
cat	16937	0	0	0	0	neg	neg
dog	16938	0	0	0	0	neg	neg
cat	16939	0	0	0	0	neg	neg
dog	16942	0	0	0	0	neg	neg
dog	16946	0	0	0	0	neg	neg
dog	16949	0	0	0	0	neg	neg
dog	16958	0	0	0	0	neg	neg
dog	16959	0	0	0	0	neg	neg
cat	16979	0	0	0	0	neg	neg
dog	17175	0	0	0	0	neg	neg
dog	17176	0	0	0	0	neg	neg
dog	17177	15	4	16	16	+	+
dog	17179	0	0	0	0	neg	neg
dog	17180	0	0	0	0	neg	neg
cat	17170	0	0	0	0	neg	neg
cat	17173	0	0	0	0	neg	neg
dog	17182	49	1	57	54	+	+
dog	17434	0	0	0	0	neg	neg
dog	17387	0	0	0	0	neg	neg
cat	17364	0	>350	2	0	+	+
dog	17386	0	0	0	0	neg	neg
dog	17390	0	0	0	0	neg	neg
dog	17310	0	0	0	0	neg	neg
dog	17403	0	0	0	0	neg	neg
dog	17453	0	0	0	0	neg	neg
dog	17458	18	30	3	7	+	+
dog	17459	0	0	0	-	neg	neg
dog	17446	0	0	0	0	neg	neg
dog	17831	0	0	0	0	neg	neg
dog	17830	0	0	0	0	neg	neg
dog	18100	0	0	0	0	neg	neg
dog	18057	1	0	0	0	neg	+
dog	17824	0	0	0	0	neg	neg
dog	17822	0	0	0	0	neg	neg
dog	17919	0	0	0	0	neg	neg
dog	17833	0	0	0	0	neg	neg
dog	17903	0	0	0	0	neg	neg
dog	17878	0	0	0	1	+	+
dog	17872	0	0	0	0	neg	neg

Table 8 (continuation) - General results for *Giardia* diagnosis, using the Study IF method ("+" - positive; "neg" - negative) and number of cysts found in each group. SVAPL diagnosis results for comparison. N=272 animals

dog	17874	0	0	0	0	neg	neg
dog	17815	0	0	0	0	neg	neg
dog	17907	0	0	0	0	neg	neg
dog	18480	0	0	0	0	neg	neg
dog	18334	0	0	0	0	neg	neg
dog	18342	77	>350	1	25	+	+
dog	18331	0	0	0	0	neg	neg
dog	18326	0	0	0	0	neg	neg
dog	18329	0	0	0	0	neg	neg
dog	18333	0	0	0	0	neg	neg
dog	18318	0	0	0	0	neg	neg
dog	18315	0	0	0	-	neg	neg
dog	18807	0	0	0	0	neg	neg
dog	18803	0	0	0	0	neg	neg
cat	18845	0	0	0	0	neg	neg
dog	18802	0	0	0	0	neg	neg
dog	18809	>300	>300	20	120	+	+
dog	18792	0	8	24	25	+	+
dog	18795	0	0	0	0	neg	neg
dog	18794	0	0	0	0	neg	neg
dog	18804	2	3	3	3	+	+
dog	19096	0	0	0	0	neg	neg
dog	19035	0	0	0	0	neg	neg
dog	19099	0	0	0	0	neg	neg
dog	19114	0	0	0	0	neg	neg
dog	19518	0	0	0	0	neg	neg
dog	19507	0	0	0	0	neg	neg
dog	19510	0	0	0	0	neg	neg
dog	19592	0	28	4	5	neg	+
dog	19516	0	0	2	4	+	+
dog	19527	0	0	0	0	neg	neg
dog	19526	0	0	0	0	neg	neg
dog	19571	0	0	0	0	neg	neg
dog	19511	0	0	0	0	neg	neg
dog	19572	0	0	0	0	neg	neg
cat	19495	0	0	0	0	neg	neg
cat	19503	0	0	0	0	neg	neg
cat	19506	0	0	0	0	neg	neg
dog	20269	0	0	0	0	neg	neg
dog	20267	0	0	0	0	neg	neg
cat	20290	0	0	0	0	neg	neg
cat	20284	0	0	0	0	neg	neg
dog	20248	0	0	0	0	neg	neg
dog	20251	0	0	0	0	neg	neg
dog	20259	0	0	0	0	neg	neg
dog	18745	0	0	2	0	+	+
dog	20272	0	0	0	0	neg	neg

Table 8 (continuation) - General results for *Giardia* diagnosis, using the Study IF method ("+" - positive; "neg" - negative) and number of cysts found in each group. SVAPL diagnosis results for comparison. N=272 animal.

dog	20262	0	0	0	0	neg	neg
dog	20710	0	0	0	0	neg	neg
dog	20715	0	0	0	0	neg	neg
dog	20748	0	0	0	0	neg	neg
dog	20749	0	0	0	0	neg	neg
dog	20763	0	0	0	0	neg	neg
dog	20769	0	0	0	0	neg	neg
dog	20757	0	0	0	0	neg	neg
dog	20780	0	0	0	0	neg	neg
dog	20816	0	0	0	0	neg	neg
dog	20818	0	0	0	0	neg	neg
dog	21222	0	0	0	0	neg	neg
dog	21223	0	0	0	0	neg	neg
dog	21227	0	0	0	0	neg	neg
dog	21236	0	0	0	-	neg	neg
cat	21242	0	0	0	0	neg	neg
cat	21268	0	0	0	0	neg	neg
dog	21385	0	0	0	0	+	neg
dog	21486	0	0	0	0	neg	neg
dog	21488	0	0	0	-	neg	neg
dog	21490	0	0	0	0	neg	neg
dog	21502	0	0	0	0	neg	neg
dog	21511	2	71	88	110	+	+
dog	21512	0	0	0	0	neg	neg
cat	21794	0	0	0	0	neg	neg
dog	21840	0	0	0	0	neg	neg
dog	21842	0	0	0	0	neg	neg
dog	21843	0	0	0	0	neg	neg
dog	21847	0	0	0	0	neg	neg
dog	21848	0	0	0	0	neg	neg
dog	21849	0	0	0	0	neg	neg
dog	21851	0	1	0	1	neg	+
dog	21854	0	0	0	0	neg	neg
dog	21856	0	0	0	0	neg	neg
dog	21915	0	0	0	0	neg	neg
dog	22274	0	0	0	0	neg	neg
dog	22276	0	3	0	0	+	+
dog	22277	0	0	0	0	neg	neg
dog	22278	0	0	0	0	neg	neg
dog	22279	0	0	0	0	neg	neg
dog	61733	0	0	0	0	neg	neg
dog	22746	0	0	0	0	neg	neg
dog	22822	0	0	0	0	neg	neg
dog	22823	0	0	0	0	neg	neg
dog	22769	91	85	21	53	+	+
cat	22753	0	2	0	29	+	+
cat	22744	0	0	0	0	neg	neg

Table 8 (continuation) - General results for *Giardia* diagnosis, using the Study IF method ("+" - positive; "neg" - negative) and number of cysts found in each group. SVAPL diagnosis results for comparison. N=272 animals.

dog	22276	0	3	0	0	+	+
dog	22277	0	0	0	0	neg	neg
dog	22278	0	0	0	0	neg	neg
dog	22279	0	0	0	0	neg	neg
dog	61733	0	0	0	0	neg	neg
dog	22746	0	0	0	0	neg	neg
dog	22822	0	0	0	0	neg	neg
dog	22823	0	0	0	0	neg	neg
dog	22769	91	85	21	53	+	+
cat	22753	0	2	0	29	+	+
cat	22744	0	0	0	0	neg	neg
dog	23017	0	0	0	0	neg	neg
dog	23024	0	0	0	0	neg	neg
dog	23025	0	0	0	0	neg	neg
dog	23037	0	0	0	0	neg	neg
dog	23217	0	0	0	0	neg	neg
dog	23218	0	0	0	0	neg	neg
dog	23250	0	0	0	0	neg	neg
dog	23291	0	0	0	0	neg	neg
dog	23212	0	0	0	0	neg	neg
dog	23337	0	0	0	0	neg	neg
dog	23303	0	0	0	0	neg	neg
dog	23278	0	0	0	-	neg	neg
dog	23336	0	0	0	0	neg	neg
dog	23328	0	0	0	0	neg	neg
dog	23333	0	0	0	0	neg	neg
dog	23326	0	0	0	0	neg	neg
dog	23273	0	0	0	0	neg	neg
dog	23323	0	0	0	0	neg	neg
dog	23285	0	3	19	9	+	+
dog	23274	0	0	0	0	neg	neg
dog	16674	0	0	0	0	neg	neg
dog	23331	0	0	0	0	neg	neg
dog	23301	0	0	0	0	neg	neg
cat	23818	0	0	0	0	neg	neg
dog	23869	0	0	0	0	neg	neg
dog	23808	0	0	1	0	neg	+
dog	23801	0	0	0	0	neg	neg
dog	23792	0	0	0	0	neg	neg
dog	23775	1	1	0	1	neg	+
dog	23773	0	0	0	0	neg	neg
dog	23798	0	0	0	0	neg	neg
dog	23854	0	0	0	0	neg	neg
dog	24634	0	0	0	0	neg	neg
dog	24617	0	0	0	0	neg	neg
dog	24638	0	0	0	0	neg	neg
cat	24577	2	15	1	19	+	+

Table 8 (continuation) - General results for *Giardia* diagnosis, using the Study IF method ("+" - positive; "neg" - negative) and number of cysts found in each group. SVAPL diagnosis results for comparison. N=272 animals.

dog	24627	30	1	10	18	+	+
dog	24977	0	0	0	0	neg	neg
dog	24979	0	0	0	0	neg	neg
dog	25020	0	0	0	0	neg	neg
dog	24993	0	0	0	0	neg	neg
dog	25026	0	0	0	0	neg	neg
dog	24975	0	0	0	0	neg	neg
dog	24998	0	0	0	0	neg	neg
cat	24985	0	0	0	0	neg	neg
dog	25194	0	0	0	0	neg	neg
dog	25203	0	0	0	0	neg	neg
dog	25209	0	0	0	0	neg	neg
dog	25210	0	0	0	0	neg	neg
dog	25211	0	0	0	0	neg	neg
dog	25218	0	0	0	0	neg	neg
dog	25520	0	0	0	0	neg	neg
dog	25533	1	0	0	0	neg	+
dog	25557	0	0	0	0	neg	neg
dog	25531	0	0	0	0	+	neg
dog	25569	71	0	14	8	+	+
dog	25535	0	0	0	0	neg	neg
dog	25554	0	0	0	0	neg	neg
dog	25566	0	0	0	0	neg	neg
dog	25489	0	0	0	0	neg	neg
dog	25490	0	0	0	0	neg	neg
dog	25536	0	0	0	0	neg	neg
dog	25524	0	0	0	0	neg	neg
dog	25568	40	32	25	38	+	+
cat	25559	0	0	0	0	neg	neg
dog	25892	16	0	27	82	+	+
dog	25920	60	71	>300	>300	+	+
dog	25901	0	0	0	0	neg	neg
dog	25893	0	0	0	0	neg	neg
dog	25899	0	0	0	0	neg	neg
dog	25918	0	0	0	0	neg	neg
dog	25876	3	1	0	0	+	+
dog	26408	0	0	0	0	neg	neg
dog	26409	0	0	0	0	neg	neg
dog	26417	0	0	0	0	neg	neg
dog	26425	0	0	0	0	neg	neg
dog	26429	0	0	0	0	neg	neg
dog	26436	0	0	0	0	neg	neg
dog	26447	0	0	0	0	neg	neg
dog	26449	>150	>>200	>150	67	+	+
dog	26468	0	0	0	0	neg	neg
dog	26472	0	0	0	0	neg	neg
cat	26467	0	0	0	0	neg	neg

Table 8 (continuation) - General results for *Giardia* diagnosis, using the Study IF method ("+" - positive; "neg" - negative) and number of cysts found in each group. SVAPL diagnosis results for comparison. N=272 animals.

cat	26484	0	0	0	0	neg	neg
dog	26775	92	82	>200	>200	+	+
dog	26789	0	0	0	0	neg	neg
dog	26780	0	0	0	0	neg	neg

Appendix II – Number of *Giardia* cysts in single scratch samples versus multi-scratch samples.

Table 9 - *Giardia* diagnosis, from dog and cat faeces, using a single scratch technique versus multi-scratch technique, having the study final results as definitive diagnosis ("neg" – negative, "+" – positive). N = 282 animals

SP	ID	Single	Multi	Diagnose
dog	22261	0	0	neg
cat	22258	0	0	neg
cat	22282	144	188	+
cat	22284	43	314	+
dog	22763	16	35	+
cat	22750	0	0	neg
cat	22755	0	0	neg
cat	24574	0	0	neg
cat	25109	12	17	+
dog	24991	19	11	+
dog	25526	19	49	+
cat	25551	16	13	+
dog	25916a	70	85	+
dog	25916b	38	>100	+
dog	25889	55	>200	+
dog	14751	0	0	neg
dog	14796	0	0	neg
dog	14774	11	97	+
dog	14628	0	1	+
dog	14289	10	19	+
dog	14020	1	1	+
cat	14030	46	>250	+
dog	15019	0	0	neg
dog	15020	0	0	neg
dog	15021	0	0	neg
dog	15023	0	0	neg
dog	15289	44	250	+
dog	15281	161	>450	+
dog	15292	0	0	neg
dog	15284	0	0	neg
dog	15583	17	82	+
cat	15599	0	0	neg
dog	15601	0	0	neg
cat	15725	0	0	neg
cat	15724	0	0	neg
dog	15678	0	0	neg
dog	15679	1	1	+
dog	15736	0	0	neg
dog	15737	0	0	neg
dog	15738	0	0	neg
dog	15763	0	0	neg
dog	15764	0	0	neg
dog	15767	0	0	neg
dog	15875	0	0	neg
cat	15877	0	0	neg
dog	15891	1	0	+
dog	15893	0	0	neg
dog	15914	0	0	neg
dog	15912	0	0	neg
dog	15911	0	0	neg
dog	15917	0	0	neg
dog	15941	0	0	neg
dog	15942	0	0	neg
dog	15958	0	0	neg
dog	15935	0	0	neg
dog	16244	0	0	neg
dog	16250	0	0	neg
dog	16278	0	0	neg
dog	16280	0	0	neg
dog	16299	0	0	neg
cat	16239	0	0	neg
cat	16251	0	0	neg
cat	16236	0	0	neg
dog	16358	0	0	neg
dog	16364	0	0	neg
cat	16931	0	0	neg
cat	16935	87	109	+
cat	16937	0	0	neg
dog	16938	0	0	neg
cat	16939	0	0	neg
dog	16942	0	0	neg
dog	16946	0	0	neg
dog	16949	0	0	neg
dog	16958	0	0	neg
dog	16959	0	0	neg
cat	16979	0	0	neg
dog	17175	0	0	neg
dog	17176	0	0	neg
dog	17177	16	16	+
dog	17179	0	0	neg
dog	17180	0	0	neg
cat	17170	0	0	neg
cat	17173	0	0	neg
dog	17182	57	54	+
dog	17434	0	0	neg
dog	17387	0	0	neg

Table 9 (continuation) - *Giardia* diagnosis, from dog and cat faeces, using a single scratch technique versus multi-scratch technique, having the study final results as definitive diagnosis ("neg" – negative, "+" – positive). N = 282 animals

SP	ID	Single	Multi	Diagnose
cat	17364	2	0	+
dog	17386	0	0	neg
dog	17390	0	0	neg
dog	17310	0	0	neg
dog	17403	0	0	neg
dog	17453	0	0	neg
dog	17458	3	7	+
dog	17446	0	0	neg
dog	17831	0	0	neg
dog	17830	0	0	neg
dog	18100	0	0	neg
dog	18057	0	0	neg
dog	17824	0	0	neg
dog	17822	0	0	neg
dog	17919	0	0	neg
dog	17833	0	0	neg
dog	17903	0	0	neg
dog	17878	0	1	+
dog	17872	0	0	neg
dog	17874	0	0	neg
dog	17815	0	0	neg
dog	17907	0	0	neg
dog	18480	0	0	neg
dog	18334	0	0	neg
dog	18342	1	25	+
dog	18331	0	0	neg
dog	18326	0	0	neg
dog	18329	0	0	neg
dog	18333	0	0	neg
dog	18318	0	0	neg
dog	18807	0	0	neg
dog	18803	0	0	neg
cat	18845	0	0	neg
dog	18802	0	0	neg
dog	18809	20	120	+
dog	18792	24	25	+
dog	18795	0	0	neg
dog	18794	0	0	neg
dog	18804	3	3	+
dog	19096	0	0	neg
dog	19035	0	0	neg
dog	19099	0	0	neg
dog	19114	0	0	neg
dog	19518	0	0	neg
dog	19507	0	0	neg
dog	19510	0	0	neg
dog	19592	4	5	+
dog	19516	2	4	+
dog	19527	0	0	neg
SP	ID	Single	Multi	Diagnose
dog	19526	0	0	neg
dog	19571	0	0	neg
dog	19511	0	0	neg
dog	19572	0	0	neg
cat	19495	0	0	neg
cat	19503	0	0	neg
cat	19506	0	0	neg
dog	20269	0	0	neg
dog	20267	0	0	neg
cat	20290	0	0	neg
cat	20284	0	0	neg
dog	20248	0	0	neg
dog	20251	0	0	neg
dog	20259	0	0	neg
dog	18745	2	0	+
dog	20272	0	0	neg
dog	20262	0	0	neg
dog	20710	0	0	neg
dog	20715	0	0	neg
dog	20748	0	0	neg
dog	20749	0	0	neg
dog	20763	0	0	neg
dog	20769	0	0	neg
dog	20757	0	0	neg
dog	20780	0	0	neg
dog	20816	0	0	neg
dog	20818	0	0	neg
dog	21222	0	0	neg
dog	21223	0	0	neg
dog	21227	0	0	neg
cat	21242	0	0	neg
cat	21268	0	0	neg
dog	21385	0	0	neg
dog	21486	0	0	neg
dog	21490	0	0	neg
dog	21502	0	0	neg
dog	21511	88	110	+
dog	21512	0	0	neg
cat	21794	0	0	neg
dog	21840	0	0	neg
dog	21842	0	0	neg
dog	21843	0	0	neg
dog	21847	0	0	neg

Table 9 (continuation) - *Giardia* diagnosis, from dog and cat faeces, using a single scratch technique versus multi-scratch technique, having the study final results as definitive diagnosis ("neg" – negative, "+" – positive). N = 282 animals

SP	ID	Single	Multi	Diagnose
dog	21848	0	0	neg
dog	21849	0	0	neg
dog	21851	0	1	+
dog	21854	0	0	neg
dog	21856	0	0	neg
dog	21915	0	0	neg
SP	ID	Single	Multi	Diagnose
dog	22274	0	0	neg
dog	22276	0	0	neg
dog	22277	0	0	neg
dog	22278	0	0	neg
dog	22279	0	0	neg
dog	61733	0	0	neg
dog	22746	0	0	neg
dog	22822	0	0	neg
dog	22823	0	0	neg
dog	22769	21	53	+
cat	22753	0	29	+
cat	22744	0	0	neg
dog	23017	0	0	neg
dog	23024	0	0	neg
dog	23025	0	0	neg
dog	23037	0	0	neg
dog	23217	0	0	neg
dog	23218	0	0	neg
dog	23250	0	0	neg
dog	23291	0	0	neg
dog	23212	0	0	neg
dog	23337	0	0	neg
dog	23303	0	0	neg
dog	23336	0	0	neg
dog	23328	0	0	neg
dog	23333	0	0	neg
dog	23326	0	0	neg
dog	23273	0	0	neg
dog	23323	0	0	neg
dog	23285	19	9	+
dog	23274	0	0	neg
dog	16674	0	0	neg
dog	23331	0	0	neg
dog	23301	0	0	neg
cat	23818	0	0	neg
dog	23869	0	0	neg
dog	23808	1	0	+
dog	23801	0	0	neg
dog	23792	0	0	neg
dog	23775	0	1	+
dog	23773	0	0	neg
dog	23798	0	0	neg
dog	23854	0	0	neg
dog	24634	0	0	neg
dog	24617	0	0	neg
dog	24638	0	0	neg
cat	24577	1	19	+
dog	24627	10	18	+
dog	24977	0	0	neg
SP	ID	Single	Multi	Diagnose
dog	24979	0	0	neg
dog	25020	0	0	neg
dog	24993	0	0	neg
dog	25026	0	0	neg
dog	24975	0	0	neg
dog	24998	0	0	neg
cat	24985	0	0	neg
dog	25194	0	0	neg
dog	25203	0	0	neg
dog	25209	0	0	neg
dog	25210	0	0	neg
dog	25211	0	0	neg
dog	25218	0	0	neg
dog	25520	0	0	neg
dog	25533	0	0	neg
dog	25557	0	0	neg
dog	25531	0	0	neg
dog	25569	14	8	+
dog	25535	0	0	neg
dog	25554	0	0	neg
dog	25566	0	0	neg
dog	25489	0	0	neg
dog	25490	0	0	neg
dog	25536	0	0	neg
dog	25524	0	0	neg
dog	25568	25	38	+
cat	25559	0	0	neg
dog	25892	27	82	+
dog	25920	>300	>300	+
dog	25901	0	0	neg
dog	25893	0	0	neg
dog	25899	0	0	neg
dog	25918	0	0	neg
dog	25876	0	0	neg
dog	26408	0	0	neg
dog	26409	0	0	neg

Table 9 (continuation) - *Giardia* diagnosis, from dog and cat faeces, using a single scratch technique versus multi-scratch technique, having the study final results as definitive diagnosis ("neg" – negative, "+" – positive). N = 282 animals

SP	ID	Single	Multi	Diagnose
dog	26417	0	0	neg
dog	26425	0	0	neg
dog	26429	0	0	neg
dog	26436	0	0	neg
dog	26447	0	0	neg
dog	26449	>150	67	+
dog	26468	0	0	neg
dog	26472	0	0	neg
cat	26467	0	0	neg
cat	26484	0	0	neg
dog	26775	>200	>200	+
dog	26789	0	0	neg
dog	26780	0	0	neg